II. REMARKS

A. Status of the Claims

Claims 1-19 were pending in the case at the time of the Office Action. Claims 2, 6-11, and 18-19 been amended in the Amendment set forth herein. Claims 1 and 12-17 have been canceled without prejudice or disclaimer. No new claims have been added.

Regarding the amendments, claim 9 has been written to be in independent form, and the remaining amended claims have been amended to depend from claim 9. Support for the amendments of the claims can be found generally throughout the specification, such as in the claims as originally filed. Thus, claims 2-11 and 18-19 are currently under consideration.

B. The Rejections Under 35 U.S.C. §103(a) Are Overcome

Claims 1-19 are rejected under 35 U.S.C. §103(a) as being unpatentable over Knight *et al.* (U.S. Patent 5,049,388; "Knight") in view of Burke *et al.* (U.S. Patent 5,552,156; "Burke"), and further in view of Waldrep *et al.* (U.S. Patent 5,958,378; "Waldrep"). Applicants respectfully traverse this rejection.

In rejecting claims under 35 U.S.C. §103, the Examiner bears the initial burden of presenting a *prima facie* case of obviousness. See *In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). In order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) the prior art reference (or references when combined) must teach or suggest all the claim limitations; (2) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (3) there must be a reasonable expectation of success. *Manual of Patent Examining Procedure* § 2142. See also *In*

re Vaeck, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991) (emphasizing that the teaching or suggestion to make the claimed combination and the reasonable expectation of success must be both found in the prior art, and not based on applicant's disclosure). It is important to note that all three elements must be shown to establish a *prima facie* case of obviousness. Thus, if one element is missing, a *prima facie* case of obviousness does not exist.

1. Knight in View of Burke and Waldrep Fails to Teach or Suggest Each Limitation of the Claimed Invention

There is no *prima facie* case of obviousness because Knight in view of Burke and Waldrep fails to teach or suggest each limitation of the claimed invention. In particular, there is no teaching or suggestion in this cited combination of references to provide for any dose of camptothecin or derivative thereof delivered via inhalation that is about 0.26 mg/m^2/day to about 1.04 mg/m^2/day. Knight makes no reference to any camptothecin or derivative of camptothecin. Burke does not teach or suggest any aerosol dose of camptothecin or derivative of camptothecin. Further, Burke does not include any in vivo data, nor does it appear to include any specific information regarding dosage of a camptothecin or derivative of camptothecin to a subject. Waldrep does not provide the missing teaching or suggestion regarding the aerosol dosage range. Waldrep pertains to high does liposomal aerosol formulations containing cyclosporin A or budesonide. It does not contain any information regarding camptothecin. Further, it does not appear to provide any teaching or suggestion pertaining to an aerosol dosage of 0.26 mg/m^2/day to about 1.04 mg/m^2/day of any drug. The Examiner, who has not cited with particularity any such teaching or suggestion, is invited to point out such teaching or suggestion.

Therefore, in the absence of any teaching or suggestion pertaining to each limitation of the claimed invention, there can be no *prima facie* case of obviousness.

2. Knight in View of Burke and Waldrep Fails to Provide Motivation to One of Ordinary Skill in the Art to Provide for the Claimed Invention

There is no *prima facie* case of obviousness based on Knight in view of Burke and Waldrep because there is no suggestion or motivation, either in the references themselves or in the knowledge of one of ordinary skill in the art, to provide for the claimed methods. Knight and Waldrep provides no teaching or suggestion for treatment of lung cancer using camptothecin. The single sentence in Knight at col. 17, lines 15-17, cited by the Examiner recites "anticancer drugs" generically, without reference to any specific anticancer agent or class of agents.

Further, Knight and Burke provides no teaching or suggestion or provide for any liposome containing DLPC. Citing col. 8, lines 31-38 of Knight and col. 6, lines 16-18 of Burke, the Examiner appears to argue that because these references recites certain specific phosphatidyl cholines, that one of ordinary skill in the art would be motivated to substitute DLPC. However, it is the Examiner's responsibility to show that some objective teaching or suggestion in the applied prior art, or knowledge generally available [in the art] would have led one of ordinary skill in the art to combine the references to arrive at the claimed invention. *Pro-Mold & Tool Co. v. Great Lakes Plastics, Intl,* 745 F.3d 1568, 1573, 37 USPQ2d 1626, 1629 (Fed. Cir. 1996). At most, the Examiner appears to impermissibly be relying on an "obvious to try" analysis.

Applicants herein attach as Exhibit A the Declaration of Dr. J. Vernon Knight (hereinafter, "the Declaration"), one of the inventors of the present patent application. Dr. Knight declares as follows:

> Around the time of filing of the first patent application pertaining to this invention, it was known in the art that each phospholipid has specific physicochemical properties. For example, it was known in the art that DLPC has a phosphate molecule to which are attached two identical acyl groups (fatty acids) each of which consists of a series of 12 carbon atoms. DLPC has a phase transition temperature of -2° C. That is the point above which the molecule becomes increasingly liquid and below which it becomes rigid. In contrast,

DMPC and DPPC with fatty acids with 14 or 16 linear carbons, respectively, have transition temperatures of 25-50° C.

Declaration, ¶5, citing to Kay, F.B., ed., Allergy and Allergic Diseases, 1996, Vol. 1, Chapter 41, p. 738; Exhibit 1 of Declaration.

Dr. Knight further notes that the degree of saturation of the fatty acid chains has an effect. A double bond (cis format) will allow the carbon chain of the fatty acid to bend which reduces the transition temperature. Double bonds (trans) that do not allow the chain to bend do not change the transition temperature (Tc). The polar head group (to which the ends of the fatty acid chain are attached) was also thought to have an effect on transition temperature (Tc), the amount depending on its configuration, but it is less well defined than the other two mechanisms. Declaration, ¶6.

In view of these differences in the chemical and physical properties of phosphatidyl cholines, a person of ordinary skill in the art would not necessarily be motivated to substitute a different phospholipid (such as DLPC) with one of the phospholipids recited in U.S. Patent 5,049,388. Declaration, ¶7. Further, one of ordinary skill in the art would have understood the statement at col. 6, lines 14-15 pertaining to use of "any lipid" in the liposomes set forth therein to not be true for aerosols, where differences in physicochemical properties of liposomes, such as length of fatty acid chains or transition temperatures, can have significant impact on the stability of liposomes.

Burke is not relevant to the present invention because it does not address aerosol formulations for delivery of any drug. Further, as discussed above, it provides no teaching or suggestion pertaining to any of the doses set forth in the presently pending claims.

Further, Waldrep provides no teaching or suggestion pertaining to treatment of cancer using any liposome aerosol composition that includes camptothecin or an analog of camptothecin

as presently claimed. Waldrep focuses on liposomal aerosols using cyclosporin and budesonide. There is no teaching or suggestion in this reference to provide for substitution of a camptothecin in the formulations. Further, as discussed above, Waldrep focuses on "high dose" liposomal aerosol formulations, and makes no mention of the specific doses recited in the pending claims.

Dr. Knight further declares that the invention set forth in the present application is partly based on his research group's discovery that DLPC incorporation into liposome particles allows for efficient aerosol delivery of drug to the lung. Declaration, ¶8. Dr. Knight notes that his group found that DLPC nebulizes efficiently at the 16-17° C operating temperature of the nebulizer, because it is in a near liquid state. *Id.* In contrast, DMPC and DPPC, with their longer fatty acid chains and higher transition temperatures, fractured in the nebulization process and released the drug which had been included in them. *Id.* The released drug collected in the bottom of the nebulizer and was useless for treatment. *Id.*

While the Examiner included claim 9 in the rejection, the Examiner failed to cite or set forth any specific teaching or suggestion, either in the cited prior art or in the knowledge of one of ordinary skill in the art, to provide for the dosage range recited in this claim. It may be that the Examiner is relying on facts within his own personal knowledge. If this is the case, then in accordance with 37 C.F.R. §1.104(d)(2), Applicants call for the affidavit of the Examiner to set forth those facts within his knowledge that form the basis of this rejection. If there is no such personal knowledge, then the rejection is based on an impermissible "obvious to try" rationale, which is not in accordance with the requirements of 35 U.S.C. §103(a).

In view of the foregoing, there is no *prima facie* case of obviousness of the claimed invention based on Knight in view of Burke and Waldrep. Therefore, it is respectfully requested that this rejection should be withdrawn.

3. The Present Methods Result in Surprisingly Superior Anticancer Efficacy

In his Declaration, Dr. Knight cites to Knight *et al.* (Anti-cancer effect of 9NC liposome aerosol on human cancer xenografts in nude mice. Cancer Chemother Pharmacol 1999;44-177-186; Exhibit 2) as a study from my laboratory which provides evidence of the surprisingly superior effect of 9-nitrocamptothecin dilauroylphosphatidylcholine liposome aerosol in treatment of human lung cancer. Declaration, ¶9. Studies were conducted in a mouse model of human lung cancer. It was found that aerosol administered material in a dosage of 76.7 ug/kg/d weekly over a period of 22 days was superior to 100 ug/kg/d injected intramuscularly five days weekly over a period of 22 days, and both treated groups were superior to untreated mice. *Id.* By day 19 tumor volumes were significantly different in all three groups ($P < 0.001$). In this study, the aerosol was administered from a nose-only nebulizer to avoid dosage from licking of coats. *Id.* On day 23, dosages were increased to 153 μg/kg/d by the aerosol route and to 200 μg/kg/d for the intramuscular route. The therapeutic effect was even more pronounced in the aerosol treatment group with the tumor volumes in the aerosol treatment receding to near normal, while the disease in the intramuscular treatment group increased progressively to the end of the experiment on day 36. *Id.*, citing to FIG. 8, page 184 of Exhibit 2.

Dr. Knight notes that in a later human trial to establish human dosage, patients with primary or metastatic lung cancer received aerosolized administration of 9-NC-DLPC liposomes for 5 consecutive days/week for 1, 2, 4, or 6 weeks followed by 2 weeks of rest to determine feasibility. Declaration, ¶10, citing to Verschraegen *et al.*, Clinical Evaluation of the Delivery and Safety of Aerosolized Liposomal 9-Nitro-20(S)-Camptothecin in Patients with Advanced Pulmonary Malignancies, Clinical Cancer Research Vol. 10, 2319-2326. April 1, 2004 (Exhibit 2 of Declaration). A partial remission was observed in 2 patients with endometrial carcinoma

metastatic to the lung only. *Id.* The remissions were noted after eight weeks of aerosol treatment. Both patients successfully underwent resection of the residual pulmonary disease and were free of pulmonary cancer 18 months later. *Id.* Thus, these results provide evidence that the present methods of treatment using aerosolized camptothecin-DLPC liposomes results in surprisingly effective anticancer activity against metastatic or primary lung cancer.

Thus, these results provide evidence that the present methods of treatment using aerosolized camptothecin-DLPC liposomes results in surprisingly effective anticancer activity against metastatic or primary lung cancer.

B. The Nonstatutory Obviousness-Type Double Patenting Rejections Are Overcome

1. The Rejection Based on U.S. Patent 6,090,407 In View of Burke and Priel

Claims 1-19 have been rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-4 of U.S. Patent 6,090,407 ("the '407 patent") in view of Burke (U.S. Patent 5,736,156) and Priel *et al.* (U.S. 5,422,344; "Priel"). Applicants respectfully traverse. Applicants traverse this rejection.

Claims 1-4 of the '407 patent pertain to a method for treating cancer by aerosol delivery of liposomes of anti-cancer drugs via a jet nebulizer, where the anticancer drug is selected from the group consisting of taxol, taxol-A, mitotane, methotrexate, mercaptopurine, lomustine, interferon, 5-fluorouracil, and etopside, wherein a final concentration of the anticancer drug in the liposomes is not greater than 5.0 mg/ml. The claims of '407 do not set forth a camptothecin, DLPC, or the aerosol dosage range recited in the pending claims of the present patent application.

The Examiner incorrectly argues that the liposomes of the '407 patent must include DLPC, even though it is not specifically claimed. At col. 2, line 34-42, the '407 patent recites:

> One specific embodiment of this object includes 9-nitrocamptothecin and dilauroylphosphatidylcholine in a ratio of about 1:10 to 1:50 wt:; with a particularly preferred embodiment having a 9-nitrocamptothecin and dilauroylphosphatidylcholine of about 1:50 wt:wt.

The specification, by reciting "one specific embodiment," makes it clear that other embodiments that do not include DLPC are contemplated. Had the specification meant to include DLPC in all embodiments, it would have recited "In all embodiments..." Various methods of producing liposomes that do not involve DLPC are set forth in the specification. See, *e.g.*, col. 2, lines 43-col. 3, line 16, which makes reference to methods utilizing lipids.

Neither Burke nor Priel discuss use of DLPC. Further, for the reasons discussed above, a person of ordinary skill in the art would not necessarily be motivated to include DLPC in the methods claimed in the '407 patent. Furthermore, neither Burke nor Priel teaches or suggests the aerosol dosage range of a camptothecin recited in the pending claims. Burke does not address aerosol administration, and Priel makes only a single reference to aerosol administration with no mention of possible dosage by aerosol.

Thus, in the absence of any teaching or suggestion as to each limitation of the claimed invention, or any motivation to one of ordinary skill in the art to provide for the claimed methods, there can be no *prima facie* case of obviousness. Therefore, it is respectfully requested that this nonstatutory obviousness-type double patenting rejection should be withdrawn.

2. The Provisional Rejection Based on Copending Application No. 10/842,977 Will Be Addressed When No Longer Provisional

Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type

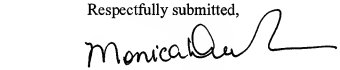
double patenting as being unpatentable over claims 1-5 of copending U.S. Patent Application 10/842,977 ("the '977 application"). The Examiner argues that although the conflicting claims are not identical, they are not patentably distinct from each other.

Applicants understand that this rejection is provisional because the conflicting claims have not been patented. Applicants will address this rejection once it is no longer provisional.

C. Conclusion

In view of the foregoing, it is respectfully submitted that each of the pending claims is in condition for allowance, and a Notice of Allowance is earnestly solicited. The Examiner is invited to contact the undersigned attorney at (512) 536-5639 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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Date: April 26, 2007

EXHIBIT A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: J. Vernon Knight, *et al.*	Group Art Unit: 1609
Serial No.: 10/663,573	Examiner: Aradhana Sasan
Filed: September 16, 2003	Atty. Dkt. No.: CLFR:158US
For: AEROSOL DRUG INHIBITION OF LUNG METASTASES	

DECLARATION OF J. VERNON KNIGHT, M.D.

I, J. Vernon Knight, hereby declare as follows:

1. I am a United States citizen residing a 29 Lana Lane, Houston, TX 77027.

2. I currently hold the position of Distinguished Service Professor, Baylor College of Medicine, Emeritus. I have expertise in the aerosol delivery of pharmaceutical agents to the lung for the treatment of lung cancer. This expertise is evidenced, for example, by my research and publications in this area (see my CV, attached as Appendix A).

3. I understand that the Examiner argues that a person of ordinary skill in the aerosol delivery of drugs to treat lung disease would be motivated to provide for liposomes containing dilauroylphosphatidyl choline (DLPC) based on the teachings of U.S. Patent 5,049,388 which discusses liposomal preparations that include other phospholipids, such as dimyristoyl-phosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC). I am the first named inventor of U.S. Patent 5,049,388. I also understand that the Examiner argues that a person of ordinary skill in the aerosol delivery of drugs to treat lung disease would be motivated to provide for liposomes containing DLPC based on the teachings of U.S. Patent 5,736,156.

4. I disagree with the Examiner's argument that a person of ordinary skill in the art would be motivated to substitute DLPC with one of the phospholipids specifically recited in U.S. Patent 5,049,388 or U.S. Patent 5,736,156.

5. Around the time of filing of the first patent application pertaining to this invention, it was known in the art that each phospholipid has specific physicochemical properties. For example, it was known in the art that DLPC has a phosphate molecule to which are attached two identical acyl groups (fatty acids) each of which consists of a series of 12 carbon atoms. DLPC has a phase transition temperature of -2° C. That is the point above which the molecule becomes increasingly liquid and below which it becomes rigid. In contrast, DMPC and DPPC with fatty acids with 14 or 16 linear carbons, respectively, have transition temperatures of 25-50° C. See discussion in Kay, F.B., ed., Allergy and Allergic Diseases, 1997, Blackwell Science Ltd, Malden, MA, Vol. 1, Chapter 41, p. 738 (Exhibit 1).

6. It is also known that the degree of saturation of the fatty acid chains has an effect on the transition temperature of the phospholipid. A double bond (cis format) will allow the carbon chain of the fatty acid to bend which reduces the transition temperature. Double bonds (trans) that do not allow the chain to bend do not change the transition temperature (Tc). The polar head group (to which the ends of the fatty acid chain are attached) was also thought to have an effect on transition temperature (Tc), the amount depending on its configuration, but it is less well defined than the other two mechanisms.

7. In view of these differences in the chemical and physical properties of phosphatidyl cholines, a person of ordinary skill in the art would not necessarily be motivated to substitute a different phospholipid (such as DLPC) with any lipid recited in U.S. Patent 5,049,388 or U.S. Patent 5,736,156

8. The invention set forth in the present application is partly based on my research group's discovery that DLPC incorporation into liposome particles allows for efficient aerosol delivery of drug to the lung. We believe that the short length of the fatty acid molecules of DLPC, which results in a reduced the transition temperature (Tc), is of major importance in stabilizing the liposome during the process of aerosolization. We found that DLPC nebulizes efficiently at the 16-17° C operating temperature of the nebulizer, because it is in a near liquid state. In contrast, DMPC and DPPC, with their longer fatty acid chains and higher transition temperatures, fractured in the nebulization process and released the drug which had been included in them. The released drug collected in the bottom of the nebulizer and was useless for treatment.

9. Knight *et al.* (Anti-cancer effect of 9NC liposome aerosol on human cancer xenografts in nude mice. Cancer Chemother Pharmacol 1999;44-177-186; Exhibit 2) is a study from my laboratory which provides evidence of the surprisingly superior effect of 9-nitrocamptothecin dilauroylphosphatidylcholine liposome aerosol in treatment of human lung cancer. Studies were conducted in a mouse model of human lung cancer. We found the aerosol administered material in a dosage of 76.7 ug/kg/d weekly over a period of 22 days was superior to 100 ug/kg/d injected intramuscularly five days weekly over a period of 22 days, and both treated groups were superior to untreated mice. By day 19 tumor volumes were significantly different in all three groups ($P < 0.001$). In this study, the aerosol was administered from a nose-only nebulizer to avoid dosage from licking of coats. On day 23, dosages were increased to 153 μg/kg/d by the aerosol route and to 200 μg/kg/d for the intramuscular route. The therapeutic effect was even more pronounced in the aerosol treatment group with the tumor volumes in the aerosol treatment receding to near normal, while the disease in the intramuscular treatment group increased progressively to the end of the experiment on day 36. See FIG. 8, page 184 of Exhibit 2.

10. In a later human trial to establish human dosage, patients with primary or metastatic lung cancer received aerosolized administration of 9-NC-DLPC liposomes for 5 consecutive days/week for 1, 2, 4, or 6 weeks followed by 2 weeks of rest to determine feasibility. A partial remission was observed in 2 patients with endometrial carcinoma metastatic to the lung only. The remissions were noted after eight weeks of aerosol treatment. Both patients successfully underwent resection of the residual pulmonary disease and were free of pulmonary cancer 18 months later. *See* Verschraegen et al., Clinical Evaluation of the Delivery and Safety of Aerosolized Liposomal 9-Nitro-20(S)-Camptothecin in Patients with Advanced Pulmonary Malignancies, Clinical Cancer Research Vol 10, 2319-2326. April 1, 2004; Exhibit 2.

11. I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date: April 26, 2007 J. Vernon Knight

J. Vernon Knight, M.D.

APPENDIX A

CURRICULUM VITAE

Name: Vernon Knight, M.D.

Date/Place of Birth: September 6, 1917, Osceola, Missouri

Marital Status: Married - 4 children

Education:

1939	A.B. William Jewell College, Liberty, MO (Humanities)
1943	M.D. Harvard Medical School, Boston, MA
1982	D.Sc. William Jewell College, Liberty, MO (Honoraris Causa)

Chronology of Employment:

BAYLOR COLLEGE OF MEDICINE

2006, June 30	Retired Emeritus
2000-2006	Baylor College of Medicine, Houston, TX Professor, Department of Molecular Physiology and Biophysics
1994-1999	Professor and Acting Chairman, Department of Molecular Physiology and Biophysics
1989-1994	Professor and Director, Center for Biotechnology
1966-1988	Professor and Chairman, Department of Microbiology and Immunology
1966-2006	Professor, Department of Internal Medicine (Infectious Disease)
1959-1966	National Institutes of Health, Bethesda, MD; Clinical Director, National Institute of Allergy and Infectious Diseases Consultant, Infectious Diseases, National Cancer, Heart and Arthritis Institutes
1954-1959	Associate Professor, Internal Medicine, Vanderbilt University Medical School
1946-1954	Cornell University Medical College - The New York Hospital, N.Y. City, Residency and Fellowship
1943	Internship, Internal Medicine, Evans Memorial Hospital, Boston University Medical Center, Department Chair, Chester Scott Keefer, M.D. In 1943 Dr. Keefer was the head of a National Research Council Committee to conduct a nationwide clinical study of the newly developed antibiotic, penicillin, in preparation for its use by U.S. Armed Forces in World War II. This activity significantly involved the house staff.

Board Certification: American Board of Internal Medicine, 1951

Military Service: U.S. Navy (MC) ETO; Jan. 10, 1944 to Sept. 25, 1946

Honors:
Distinguished Faculty Award, 1987, Baylor
Distinguished Service Professor, 1986, Baylor
Guy R. Odum, Jr. Award, M.D. Anderson Hospital, 1986
The Kyle and Josephine Morrow Distinguished Professor of Microbiology and Immunology, 1984, Baylor
Honorary D.Sc., William Jewell College, Liberty, MO, 1982

Patents Issued:

Baylor College of Medicine

1. "Small Particle Aerosol Generator for Treatment of Respiratory Disease Including the Lungs"
   a. Inventor - Vernon Knight, M.D.
      Samuel Z. Wilson
   b. U.S. Patent No. 4,649,911 , granted 1987

   This patent was the basis for development of the widely used ribavirin aerosol treatment of respiratory syncytial virus infection in babies.

Clayton Foundation of Texas

1. The Foundation's seminal Knight, et al. patent is U.S. Pat. No. 5,049,388 granted September 17, 1991, for "Small Particle Aerosol Liposome and Liposome-Drug Combinations for Medical Use". This patent disclosed fundamental aerosol processes developed by Dr. Knight and his colleagues, and it has spawned numerous other inventions.
2. U.S. Patent No. 5,958,378 granted September 28, 1999, for "High Dose Liposomal Aerosol Formulations Containing Cyclosporin A or Budesonide"
3. U.S. Patent No. 6,440,393 granted August 27, 2002, for "Carbon Dioxide Enhancement of Inhalation Therapy" An application for reissue of this patent is pending, an objective of which is to provide more specific values for carbon dioxide in the claims.
4. U.S. Published Pat. Appl. No. 20020106330 published August 8, 2002, for the same invention as (3) above.

   The next five listings are patents and applications for cancer indications.

5. U.S. Patent No. 6,090,407 granted July 18, 2000, for "Small Particle Liposome Aerosols for Delivery of Anti-Cancer Drugs" (Taxol among others).
6. U.S. Patent No. 6,346,233 granted February 12, 2002, for "Composition for Treating Cancer Via Liposomal Aerosol Formulation Containing Taxol"
7. U.S. Published Patent Appl. No. 20020102296 published August 1, 2002, for "Small Particle Liposome Aerosols for Delivery of Anti-Cancer Drugs" (camptothecins among other things).
8. U.S. Published Patent Appl. No. 20040208935 published October 21, 2004, for "Small Particle Liposome Aerosols for Delivery of Anti-Cancer Drugs" (camptothecins and others).

9. U.S. Published Patent Appl. No. 20030215494 published November 20, 2003, for "Aerosol Drug Inhibition of Lung Metastases"

In varying ways, the next five patents and applications relate to gene therapies.

10. U.S. Patent No. 6,106,859 granted August 22, 2000, for "Stabilization of Lipid: DNA Formulations During Nebulization"
11. U.S. Patent No. 6,375,980 granted April 23, 2002, a continuation-in-part of the patent in (10) above.
12. U.S. Patent No. 6,656,916 granted December 2, 2003, for "Glucocorticoid Enhancement of Gene Expression"
13. U.S. Published Patent Appl. No. 20040028616 published February 12, 2004, for "Inhibition of Lung Metastases by Aerosol Delivery of p53 Gene and Anti-Cancer Compounds"
14. U.S. Patent Appl. No. 09/540,916 filed March 31, 2000, for "Polyethyleimine: DNA Formulations for Aerosol Delivery" (This is a pending application that either has not been published, or we simply weren't able to find it in the Patent Office's on-line database)

The following two pending patent applications relate to Dr. Knight's very able collaborations with Foundation researchers outside of Baylor College of Medicine.

15. Published Patent Appl. No. 20030236301 published December 25, 2003, for "Liposomal Delivery of Vitamin E based Compounds" (Dr. Knight's collaborators/co-inventors are at The University of Texas at Austin.
16. U.S. Published Patent Appl. No. 20050181036 published August 18, 2005, for "Aerosol Delivery of Curcumin" (Dr. Knight collaborator/co-inventor is at The University of Texas M.D. Anderson Cancer Center)

Finally, Dr. Knight is not names as a co-inventor in the next patent, but the invention arose under his direction and in his laboratory.

17. U.S. Patent No. 6,334,999 granted January 1, 2002, for "Liposomal Aerosols for Delivery of Chemotherapeutic Retinoids to the Lungs"

Appointments:

The Methodist Hospital, Houston	Senior Attending Physician, Emeritus 1966- Medical Staff Executive Com. 1981-1995
The Methodist Hospital Foundation	Chairman, Grants Awards Committee, 1986-
M.D. Anderson Cancer Center Houston, TX	Consultant in Infectious Disease Dept. of Developmental Therapeutics 1976-2000
Ben Taub General Hospital Houston, TX	Attending Physician 1966-

Veterans Administration Hospital	Deans Committee and Physician/Consultant, 1966-
Viratek, Inc. Costa Mesa, CA	Board of Directors 1981-1995
ICN Pharmaceuticals, Inc. Costa Mesa, CA	Board of Directors 1995
ZymeTx Oklahoma City, OK	Board of Directors 1996-2001
National Institutes of Health	National Advisory Allergy and Infectious Diseases Council 1975-1978
American Institute of Biological Sciences, Arlington, VA	Medical Sciences Review Panel to NASA 1976-1980
U.S. Army Medical Research Institute of Infectious Diseases Ft. Detrick, Frederick, MD	Professional Consultant 1962-1981
Association of Medical School Microbiology Chairman	President 1981
Contemporary Arts Museum Houston, TX	Board of Directors 1977-1982
Gorgas Memorial Institute of Tropical and Preventative Medicine, Washington, D.C	Board of Directors 1977-1991
Genetic Engineered Systems, Inc. The Woodlands, TX	Chairman of the Board 1989-1990

Scientific and Clinical Board Appointments:

Houston Biotechnology Incorporated The Woodlands, TX	Scientific and Clinical Advisory Board-1989
Woodlands Venture Capital Group The Woodlands, TX	Scientific Advisory Committee- 1989
Zonagen Incorporated The Woodlands, TX	Scientific Advisory Committee- 1989

Professional Societies

Association of American Physicians
American Society for Clinical Investigation
AOA, Beta Chapter
American Clinical and Climatological Association
American College of Physicians
American Federation for Clinical Research
American Society for Microbiology (National and Texas branch)
Association of Medical School Microbiology Chairmen
Harris County Medical Society
Infectious Diseases Society of America (emeritus)
International Association of Aerobiology
International Leprosy Association
New York Academy of Sciences
Society for Experimental Biology and Medicine
Society of Sigma XI
Texas Medical Association

Directory Listings:

Who's Who in America
American Men of Science

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EXHIBIT 1

Allergy and Allergic Diseases

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CHAPTER 41

New Approaches in Aerosol Drug Delivery for the Treatment of Asthma

V. Knight & J.C. Waldrep

Introduction

Aerosols are dispersions in air of solid or liquid particles, of fine enough particle size, and consequent low enough settling velocities, to have relative airborne stability. In quiet air, a spherical unit density particle of 10 μm diameter would take 17 minutes to settle the height of a room 3 m). A 1 μm particle would take 24 hours to settle this distance (Knight, 1973).

Aerosols have been widely and beneficially used for many years for delivery of drugs for the treatment of asthma. The treatment nevertheless has limitations, resulting from the exceptional efficiency of the human respiratory tract to limit the penetration of inhaled particles to the lower respiratory tract and to remove particles deposited in upper respiratory sites by mucociliary action and swallowing. It is probably a manifestation of these difficulties that there are myriad devices on the market, each seeking to improve efficiency of pulmonary deposition of inhaled particles. These include the widely used metered dose inhalers (MDI) of various configurations and dry-powder inhalers (DPI). Recently, the problem has been further complicated by the requirement that the use of chlorofluorocarbon (CFC) propellants, the most widely used methodology, must be discontinued because of environmental hazard. There is a major effort now under way to develop alternative methods of aerosol treatment, but the technology by which this will be achieved has not yet emerged. In this chapter, we shall review both traditional and newly developed technologies applicable to the treatment of asthma.

General considerations of particle deposition in the respiratory tract

The advantages of aerosol delivery of drugs to the respiratory tract are that the drug is deposited on diseased lung surfaces immediately after start of treatment and the total dose required for treatment is generally much lower than that required by oral or intravenous routes. Drugs deposited in the respiratory tract are cleared by macrophages or slowly enter the circulation directly or through lymphatics. Many drugs are metabolized to some extent in the lung as well (Andersson & Ryerfeldt, 1984; Debs, 1990). When these effects are combined with the low total dose, concentrations in serum and organs other than the lung are low and high peak levels that may cause systemic toxicity are avoided. The rate of clearance from the lung is influenced by biochemical properties of the compound, such as solubility, lipophilicity and molecular size (Schanker *et al.*, 1986; Byron & Patton, 1994). Water-soluble drugs are absorbed more slowly than lipid-soluble drugs (Schanker *et al.*, 1986). Absorption is greatest in the alveolar area, which constitutes the largest absorbing surface in the respiratory tract. The clearance of both lipid-soluble and water-soluble drugs is reduced substantially by incorporation into liposomes.

A disadvantage of aerosol treatment is the loss of aerosol that is produced in excess of the patient's capacity to inhale it and the exhalation of inhaled aerosol that is not deposited. In addition, with nasal breathing, large numbers of particles deposit in the nose and are promptly cleared by mucociliary action to the pharynx where they are swallowed. Nasal deposition constitutes essentially oral dosage and may present a risk of sys-

temic toxicity when powerful agents such as glucocorticoids (GC) are used. Drugs deposited in the tracheobronchial area are transported upward to the pharynx by mucociliary action, where they are swallowed. The larger bronchi do not constitute a large surface area and thus their burden of deposited particles is small. Larger numbers of particles deposit in the more peripheral and more extensive smaller airways. They, too, are transported upward through mucociliary action, but the process is much slower, allowing time for local drug action. The most peripheral and most extensive portion of the lungs, consisting of partially and fully alveolated airways, do not possess the mucociliary system and clearance from these sites is primarily into the circulation.

From the foregoing, it is evident that avoiding nasal deposition by using mouth breathing would be advantageous. Greater efficiency of treatment would result if aerosol could be supplied to the patient only during inspiration. This methodology is currently available and details of its use will be presented. A drawback to mouth breathing is patient discomfort when long periods of treatment are required, but this is not a problem in asthma therapy.

As the use of aerosol treatments increases with a greater variety of drugs, adjuvants of aerosol delivery, such as liposomes or other carrier particles, and the use of different kinds of devices to generate aerosols are being studied. A method that can be used to make comparisons among these potentially numerous delivery systems would be advantageous. In the past, model systems resembling the human respiratory tract have also been used successfully. There are logistic reasons, however, that discourage their widespread use.

We propose an alternative system for valuating aerosols for human treatment, namely, calculation of regional deposition of inhaled aerosol based primarily on particle size but also including quantitative deposition, so that precise comparisons among aerosol generators can be made (Lippman *et al.*, 1980; Persons *et al.*, 1987a,b). Such a method could compare the output from jet nebulizers, MDI, DPI and other devices. The experimental and theoretical basis of particle deposition is substantial, and reasonable approximations of regional deposition of aerosols are possible.

The expanding use of aerosols has also increased the need to know which pattern of pulmonary deposition is best for the treatment of particular disease entities. While the disease, asthma, is most conspicuous because of its interference with small airway function, the most efficient and effective site of aerosol treatment for it has not been clinically defined. In the following section, we shall describe a lung model which uses particle size, drug content and other variables to predict regional dosage of inhaled particles.

Calculation of drug delivery by aerosol

While the predominant sites of deposition of particles generated by propellants under pressure, as with MDI or DPI, or droplets generated from liquid suspensions by jet nebulizers is determined principally by the size of the particles or droplets, some particles throughout the size range will be deposited throughout the respiratory tract. The generalization can be made that the largest particles will deposit in nose, throat and bronchial passages and the smallest particles will deposit principally in the lung periphery.

This process can be defined more precisely by relating it to Weibel's anatomical model of the human lung (Weibel, 1963). Above the larynx, most particle deposition, by far, occurs in the nose. Figure 41.1 shows the Weibel model of the lung, in which the conducting airway generations are numbered 0–16 from the trachea to the fine bronchioles. As stated earlier, these areas are ciliated and contain mucus-secreting cells to form the mucociliary system, which propels deposited particles proximally to be eventually swallowed. Generations 17–23 are increasingly alveolated and do not contain the mucociliary apparatus, but they constitute the largest portion of the lung surface.

If nasal deposition is excluded from consideration, as is proposed for the treatment of asthma, calculated deposition of inhaled particles in the lung, with mouth breathing, according to a range of particle sizes is shown in Table 41.1. We believe that, for asthma treatment, deposition in the mouth and upper generations of the conducting airways should also be minimized, and this can be best achieved with a particle size of about 1.6 μm mass median aerodynamic diameter (MMAD). Such particles deposit principally in the alveolated lung area, but a significant amount will deposit in the 0–16 generations. We believe, also, that deposition in these lung areas will be suitable for the treatment of asthma; however, data describing other deposition patterns according to particle size are shown. Major factors which determine site of particle deposition include inertial impaction, occurring principally in the nasopharynx and upper airways, where the rate of air flow is greatest. Sedimentation in smaller airways is principally due to gravity. Particles <0.5 μm MMAD, however, deposit in terminal airways by gaseous diffusion (Brain *et al.*, 1985; Kohler & Fleisher, 1991). Obviously, the size and configuration of the respiratory tract in health and disease will further affect particle deposition. As a generalization, any process which distorts the airway or reduces its diameter will cause increased deposition of particles.

Hygroscopicity influences deposition, due to increase in size of droplets as they progress down the warm and humid passages of the respiratory tract. This effect is most

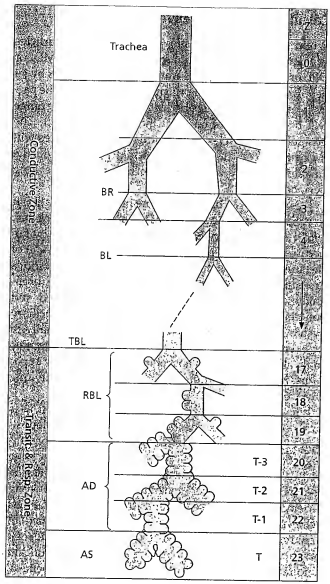

Fig. 41.1 Diagrammatic representation of the sequence of elements in the conducting and transitory zones of the airways leading to terminal alveolated lung spaces. (From Weibel, 1963.)

conspicuous with nasal breathing, during which 35–70% of inhaled hygroscopic particles of 2–6 μm MMAD will deposit in the nose (Knight, 1973). With mouth breathing, which bypasses the nose, there is little difference in deposition patterns of non-hygroscopic and a range of aerosols of increasing hygroscopicity (Table 41.2). Increasing tidal volume (Table 41.3) causes increased peripheral lung deposition, because of deeper penetration and longer residence time of particles in the lung. Breath holding (Table 41.4) is associated with an even greater effect on peripheral lung deposition. It has been found difficult, however, to employ these procedures in patients who are ill.

Asthma has a large impact on children; because of their greater need of respiratory gas exchange to provide for metabolic and growth requirements, individual dose calculations based on age are required. Table 41.5 shows that, when breathing the same aerosol over the same period of time as an adult, a 6-year-old child will deposit 1.9 times as many particles as the adult per kilogram of body

Table 41.1 Effect of particle size on regional aerosol particle deposition* (tidal volume 750 ml, geometric standard deviation (GSD) 2.0). (Data provided by Keyvan Keyhani, Department of Bioengineering, University of Pennsylvania, Philadelphia, PA.)

MMAD (μm)	Mouth (%)	0–16 (%)	17–23 (%)	Total (%)
0.5	0.2	1.7	11.6	13.5
1.0	2.0	4.6	17.6	24.3
3.0	10.1	11.0	22.7	44.1
6.0	29.5	20.0	18.1	68.0
9.0	41.9	22.3	11.7	77.3

* Regional deposition of inhaled particles according to the Weibel lung model (Weibel, 1963). Breathing characteristics assumed for an adult were functional residual capacity 3300 ml; mouth breathing, inspiration 2 seconds; expiration 2 seconds; no breath holding.

Table 41.2 Effect of salt concentration on regional aerosol particle deposition* (tidal volume 750 ml, MMAD 1.6 μm, GSD 2.0).

Salt concentration	Mouth (%)	0–16 (%)	17–23 (%)	Total (%)
Non-hygroscopic	3.8	9.3	20.7	33.8
0.0045 g/ml (½ normal)	2.8	4.6	17.6	24.3
0.0090 g/ml (normal)	2.1	6.2	20.9	29.1
0.0180 g/ml (2× normal)	2.2	8.2	24.1	34.5

* Footnote as in Table 41.1

Table 41.3 Effect of tidal volume on regional aerosol particle deposition* (MMAD 1.6 μm, GSD 2.0). (Data from Keyvan Keyhani, Department of Bioengineering, University of Pennsylvania, Philadelphia, PA.)

Tidal volume (ml)	Mouth (%)	0–16 (%)	17–23 (%)	Total (%)
1000	3	4.3	20.1	28.1
750	2	4.6	17.6	24.2
500	1.2	5.5	11	17.6

* Breathing characteristics as in Table 41.1, except for tidal volumes.

Table 41.4 Effect of breath holding on regional aerosol particle deposition* (tidal volume 750 ml, MMAD 1.6 μm, GSD 2.0). (Data from Keyvan Keyhani, Department of Bioengineering, University of Pennsylvania, Philadelphia, PA.)

Breath holding time (seconds)	Mouth (%)	0–16 (%)	17–23 (%)	Total (%)
0	2.01	4.63	17.6	24.3
2	2.04	7.12	35.5	44.7
10	2.21	10.9	53.4	66.5
20	2.26	13.9	60.4	76.8

* Breathing characteristics as in Table 41.1 except for breath holding.

Table 41.5 Respiratory tract deposition of aqueous aerosol particles in intubated patients according to age (MMAD 1.4 μm, GSD 1.6).

Age (years)	0–16 (%)	17–23 (%)	Total (%)	Relative dose* (mg/kg)
6	19	55	18	
8	21	54	17	
12	22	53	13	
16	24	53	10	
25	24	52	10	

* Adjusted for age-related differences in ventilation; 25 years' ventilation = 1, based on aqueous ribavirin aerosol (Knight *et al.*, 1988).

weight. This value is 1.7, 1.3 and 1.0 for children of ages 8, 12 and 16. Dosage calculations for children should take these factors into account. In contrast to total deposited dose, there is little difference in regional deposition between children and adults (Table 41.5).

Currently available drug aerosol delivery systems

MDI

In recent years, aerosol administration of asthma medications has been dominated by the widespread usage of MDI. MDI have been developed and effectively utilized with different bronchodilators (e.g. albuterol), antiallergics (e.g. nedocromil sodium) and GC. Inhaled GC are a very effective treatment of asthma (Reed, 1991; Szefler, 1992; McFadden, 1993; Toogood, 1993). These topically active GC have minimal effects on the hypothalamic–pituitary–adrenal axis, except when daily doses of 1000 μg or more are employed. The development of topically potent GC with first-pass liver metabolism has minimized systemic side-effects (Ryrefeldt *et al.*, 1982; Andersson & Ryrefeldt, 1984; Brattsand *et al.*, 1992). However, oropharyngeal complications of candidiasis (localized inhibition of host defences by GC) and dysphonia (toxic response to CFC propellants) are more common with MDI (Toogood *et al.*, 1980; Toogood, 1993). The development of spacer extensions or holding chambers has been necessitated by these complications (Toogood *et al.*, 1984).

However, environmental concerns and the 1996 worldwide ban on CFC propellants have caused manufacturers to phase out CFC production and have necessitated the development of alternative propellants and devices (Newman, 1990; Balmes, 1991). Most of the propellants in development for current MDI devices are not compatible with existing metering valves, excipients and some manufacturing components, and the toxicology issues remain to be delineated. Complex interactions may occur between the newer propellants, surfactants and drugs (Niven, 1993). Furthermore, contribution to the greenhouse effect by the newer propellants could lead to regulated utilization (Martin *et al.*, 1994).

MDI may be unsuitable in some patients, such as infants, children, the elderly or the chronically ill (O'Doherty & Miller, 1993). Furthermore, the required coordination between actuation and inhalation may result in inadequate aerosol drug delivery from MDI in many patients (DeBlaquiere *et al.*, 1989; Manzella, 1989; Hilton, 1990). Much of the aerosolized drug from the MDI is lost in the device or deposited in the upper part of the respiratory tract. Particle sizes produced by MDI are generally large and the deposition patterns can be reasonably well predicted from data supplied in this chapter. Table 41.6 shows droplet size produced by several commercially available MDI. The average particle size is calculated at 4–5 μm MMAD with a geometric standard deviation (GSD) of > 2. By interpolation from our computer model of mouth breathing, the deposition pattern at 4.8 μm MMAD would be about 20% deposited in mouth, 15% in 0–16 generations and 21% in 17–23 generations. Some estimates, however, put the lung deposition value at approximately 10% (Newman *et al.*, 1981).

DPI

In response to the above concerns regarding MDI, alternative delivery systems, such as the DPI, have been developed. The complex design features of DPI rely on the patient's inspiratory flow to generate and deliver drug aerosols. DPI have been developed for the aerosol delivery of β_2-agonists, bronchodilators, sodium cromoglycate and some GC (Timsina *et al.*, 1994). Suitable DPI formulation requires that the powdered drug be available in a stable, bioactive form (Niven, 1993). The performance of DPI is determined, in part, by powder formulation,

Table 41.6 Aerodynamic size distribution of metered dose inhaler aerosols. (Condensed from Kim *et al.*, 1985.)

Tade name	Generic name	MMAD (90% RH) (μm)	GSD
Duo-Medihaler	Isoprenaline	5.7	2.3
	Phenylephrine		
Medihaler ISO	Isoprenaline	4.1	2.0
Medihaler EPI	Adrenaline	5.2	2.2
Alupent	Orciprenaline	5.3	2.1
Proventil	Albuterol	2.6	2.3
Bronkometer	Isoetharine	5.7	2.4
	Phenylephrine		
Mistometer	Isoprenaline	3.9	2.2
Beclovent	Beclomethason	4.0	2.1
Aristocort	Triamcinolone Acetonide	4.7	2.4

See text for definition of abbreviations.

including the carrier powder. The effects of formulation variables on pulmonary deposition of DPI aerosols, surface properties of the carrier, optimum carrier size, drug to carrier ratio, relative humidity, electrostatic behaviour, the use of tertiary components and process conditions are poorly characterized (Martin *et al.*, 1994; Timsina *et al.*, 1994).

Some carriers have induced irritation, coughing or bronchoconstriction (Timsina *et al.*, 1994). An important variable in DPI therapy is the required energy input during inspiration for deaggregation of drug particles from carrier. Forces between drug and carrier must be sufficient to ensure that there is no deaggregation during filling and handling, but to allow extensive detachment when subjected to the turbulent air flow generated within the device (Martin *et al.*, 1994). While DPI are clinically effective in certain types of patient, the breath-actuated output is inefficient and highly variable, delivering a pulmonary dose of about 10% (Niven, 1993). Resistance to air flow through various DPI devices is a problem in some asthmatics, children and the elderly and is dependent on age, sex, height and disease state (Martin *et al.*, 1994; Timsina *et al.*, 1994). The main advantages of DPI are drug delivery co-ordinated with inhalation, environmental safety and low cost (Martin *et al.*, 1994; Olsson & Asking, 1994).

Continuous-flow jet nebulizers

Continuous-flow jet nebulizers have been utilized in asthma for aerosol delivery of different water-soluble medications (bronchodilators, e.g. albuterol) and a few micronized GC suspension formulations (beclomethasone dipropionate (Bec) and budesonide) (Bisgaard, 1994; Wood & Knowles, 1994). Nebulizer-based aerosol therapy is on the increase worldwide, particularly in Europe. The clinical utilization of nebulizers has proved to be particularly successful for hospital and non-ambulatory settings, as well as paediatric and geriatric patients (Dalby & Tiano, 1993). More efficient, convenient, portable and disposable nebulizers and compressors will be utilized with increased regularity in the future (Dalby & Tiano, 1993). A major limitation in this form of aerosol therapy has been the paucity of suitable formulations. There are several important factors which affect therapeutic efficacy of aerosol formulations delivered by nebulizers. Nebulizer design, operating conditions (e.g. flow rate) and ancillary equipment (tubing, connectors, mouthpiece, face masks) are important variables which must be standardized (Dalby & Tiano, 1993). Nebulizer variation, either within manufactured lots or among devices, is an important variable affecting aerosol output efficiency and aerodynamic properties (O'Doherty, 1993). The composition of the aerosol preparation will also influence aerosol output efficiency and aerodynamic properties (Waldrep *et al.*, 1993, 1994c). For example, formulations of liposomes developed for intravenous or other delivery routes are generally not suitable for aerosolization, due to instability or inappropriate biophysical properties (Waldrep *et al.*, 1993, 1994a,c). To achieve the best lower-pulmonary deposition with minimized systemic delivery, aerosols should contain particles less than 5 μm MMAD (ideally between 1 and 3 μm). Many of the currently available jet nebulizers produce aerosols in this size range (Waldrep *et al.*, 1993, 1994c). The operating parameters (e.g. flow rate) must be strictly defined and tested for each device with each standardized formulation (Dalby & Tiano, 1993; Waldrep *et al.*, 1994b).

Alternative aerosol therapy for asthma: drug–liposomes

Aerosol delivery of water-insoluble, hydrophobic compounds currently available for the treatment of asthma has been limited. The recent development of liposomal formulations compatible with aerosol delivery has expanded the potential for more effective utilization with additional drugs and has many potential advantages including: aqueous compatibility, sustained pulmonary release to maintain therapeutic drug levels and facilitated intracellular delivery (particularly to alveolar macrophages) (Schrier *et al.*, 1993). The half-life of drug–liposomes in the lung is significantly longer than that of soluble formulations (Juliano & McCullough, 1980). Liposomes are retained for an even longer period; for example, 50–60% of phosphatidylcholine (PC) lipo-

omes may be retained for 24 hours after inhalation (Morimoto & Adachi, 1982; Pettenazzo *et al.*, 1989; Vidgren *et al.*, 1994). Inhaled liposomes delivered to the terminal airspaces associate rapidly with the alveolar surfactant and enter the intracellular phospholipid pool without altering normal metabolism or macrophage activity (Mihalko *et al.*, 1988). In addition, phospholipase-mediated hydrolysis is minimal (Mihalko *et al.*, 1988). These results suggest that drug–liposome aerosols should be more effective for delivery, deposition and retention of water-insoluble, hydrophobic, lipophilic compounds, in contrast to water-soluble compounds (Niven & Schrier, 1990; Taylor *et al.*, 1990; Taylor & Farr, 1993). Drug–liposome aerosol technology is readily adaptable to conventional or alternative asthma drugs not currently available for aerosol treatment (Szefler, 1992). The highly potent, extremely lipophilic GC currently employed in MDI/DPI for aerosol-based asthma therapies are well suited for liposome aerosol formulation (Waldrep *et al.*, 1993; 1994b,c). In addition, a more innovative development is the use of cyclosporin A (CsA) liposome aerosol for the treatment of asthma (and other immunologically mediated pulmonary diseases) (Gilbert *et al.*, 1993; Waldrep *et al.*, 1993).

In general, hydrophobic drugs incorporate into liposome membranes and hydrophilic drugs are entrapped in the aqueous vesicles of liposomes (New *et al.*, 1990). Large or intermediate-sized multilamellar liposomes are best for use with hydrophilic drugs because of their greater volume; however, hydrophilic compounds are lost in appreciable amounts from liposomes as a result of nebulization (Niven *et al.*, 1990). Biophysical properties of liposomes are influenced by both drug and lipid composition. Nuclear magnetic resonance (NMR) spectroscopy has proved to be a useful tool for determining the orientation of drugs and cholesterol within the liposomal bilayers (Garcon *et al.*, 1989). GC may interact with phospholipid bilayers as its structural analogue, cholesterol. Association of other more hydrophobic drugs, like CsA, in liposomal bilayer or aqueous compartment varies with the phospholipid composition of the liposome (Stuhne-Sekalec *et al.*, 1988, 1991). With liposomal formulations of many hydrophobic drugs (like some GC and CsA), there is a stable association which withstands the shear forces generated during nebulization, reflux and recirculation within the nebulizer reservoir. However, Gilbert *et al.* (1988) and Farr *et al.* (1985) demonstrated significant size reduction in multilamellar vesicle (MLV) liposomes within the nebulizer reservoir resulting from shearing during nebulization. In aerosol it is the aqueous droplet and not the liposome that determines particle size distribution (Farr *et al.*, 1985; Niven *et al.*, 1990; Taylor *et al.*, 1990; Waldrep *et al.*, 1993).

Aerosol droplets may contain none, one or more drug–liposome particles. Aqueous droplets containing lipid particles resist reduction in MMAD in conditions of decreasing relative humidity (RH) until RH reaches about 70% from 95% (Marks *et al.*, 1983). In contrast, hygroscopic saline aerosol droplets shrink in size stepwise with reducing RH. As noted earlier, hygroscopic effects have little effect on respiratory tract deposition of aerosols inhaled through the mouth.

Characteristics of liposome aerosols produced with commercially available jet nebulizers

In a recent study, the MMAD, GSD and aerosol output of 18 commercially available jet nebulizers was determined using Bec–liposomal formulation (Waldrep *et al.*, 1994b). Figure 41.2 shows the estimated percentage regional deposition of inhaled aerosol droplets within Weibel lung generations 0–23. Nebulizers were ranked by increasing value with estimated percentage deposition approximating 25% for generation 17–23 and ranging from 5 to 8% in generations 0–16. Mouth deposition was calculated at about 5%. Thus, there is a considerable degree of uniformity of droplet distribution according to size among these randomly selected, commercially available nebulizers. Differences in output can be attributed to different flow rates employed to generate aerosols as well as design differences (Fig. 41.3). Thus, while MMAD and GSD are

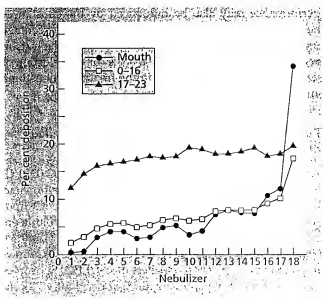

Fig. 41.2 Calculated percentage deposition from 18 nebulizers of beclomethasone dipropionate (Bec) contained in Bec–dilauroyl PC (DLPC) liposome aerosol in mouth, Weibel generations 0–16 and 17–23. The reservoir contained 0.5 mg/ml of Bec in liposomes. Nebulizers 1–18 were Up Mist, Power Mist, Hudson Hand Held, Nb Mist, Ava Neb, Respirgard, Acorn, Whisper Jet, Aqua Tower, Permanent Neb, Custom Neb, Pari-Jet, Puritan Single Jet, Raindrop 3040, SPAG, Spira and Heart. (From Waldrep *et al.*, 1994b.)

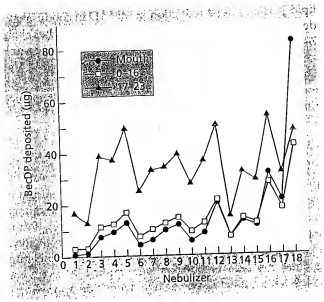

Fig. 41.3 Weight in micrograms of Bec calculated to deposit on regional sites as described in Fig. 41.2. (From Waldrep *et al.*, 1994b.)

major determinants of the site of deposition of aerosol particles within the respiratory tract, the amount of drug delivered is a function of the nebulizer design and operation. The results of this study suggest that there is a wide range of operating characteristics among nebulizers, and performance may also be influenced by the substances nebulized. MMAD, GSD and the predicted percentage regional deposition of drug–liposomes within the human respiratory tract could provide a basis for the selection of nebulizers best suited for use in the treatment of asthma and other inflammatory lung diseases.

Pulmonary deposition of technetium-99m-labelled drug–liposome aerosols

A gamma-labelling method for Bec–dilauroyl PC (DLPC) liposomes has been developed to monitor deposition and mucociliary clearance of aerosolized Bec-DLPC liposomes, and this has been performed in normal volunteers (Vidgren *et al.*, 1994). Plate 41.1 (opposite page 524) shows pulmonary deposition patterns in two individuals following inhalation of ^{99m}Tc-labelled Bec–DLPC liposomes (anterior scan). Lung scans were performed after 20 consecutive inhalations from breath-actuated nebulizers: (A) using an Aerotech II nebulizer with Bec–DLPC liposome aerosol output of MMAD 1.5 µm and GSD 2.4; and (B) using a Spira nebulizer with Bec–DLPC liposome aerosol output of MMAD 3.6 µm and GSD 2.5. Pulmonary scans were performed immediately after inhalation and at 1, 2 and 3 hours post-inhalation. In the A series, scintigraphs of pulmonary radioactivity displayed both central and peripheral deposition patterns, which remained nearly constant (some loss due to decay is apparent). Some accumulation of radioactivity was noted in the stomach at 0 and 1 hours. In contrast, in B, pulmonary radioactivity was initially less, with more central deposition. Lung clearance proceeded more rapidly and activity in the stomach was substantially greater throughout the period of observation.

Figure 41.4 demonstrates pulmonary radioactivity in a similar study following inhalation of a ^{99m}Tc-labelled liposome preparation (Farr *et al.*, 1985). The size of the inhaled liposome aerosol droplets was 3.7 µm MMAD and GSD 1.5. Greatest radioactivity was localized centrally, presumably associated with the conducting airways, with much less activity detected in the periphery of the lung. This distribution resembles that in Plate 41.1B, in which the droplet size of the inhaled liposome aerosol was similar, at 3.6 µm MMAD. Observations in both of these studies are consistent with patterns of deposition predicted by our computer model for aerosols containing these droplet sizes. Although drug–liposome aerosols may be less hygroscopic than aerosols of aqueous drugs, the difference is probably not great, and it is a reasonable assumption that drug–liposome aerosol deposits within

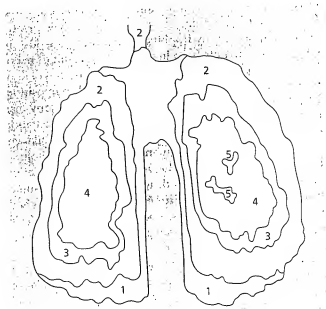

Fig. 41.4 Diagnostic representation of gamma-activity contours obtained from a typical scintigram. Posterior view immediately after inhalation of ^{99m}Tc-labelled liposomes, MMAD 3.7 µm, GSD 1.5. Areas marked 1–5 represent increasing intensity of activity. Note values of 4 and 5 in the hilar region of both lungs, an area corresponding with the first few Weibel generations of conductive airway branchings. (From Farr *et al.*, 1985.)

the lung in a manner analogous to that of 0.5 normal saline (0.0045 g/ml NaCl).

Tolerance and safety of liposome aerosols

Toxicity studies have been performed in animals and humans after inhalation of empty liposomes. Myers *et al.* (1993) exposed mice for 4 weeks to inhalations of commercially available hydrogenated soybean phosphatidylcholine (HSBPC) in concentrations approximating those that might be used for human treatment. A variety of observations, including lung histology, fatty acid analysis of lung tissue, macrophage morphology, phagocytic function, weight gain, and physical appearance, showed no untoward effects of aerosol treatment. Thomas *et al.* (1991) exposed 10 volunteers to a 1-hour aerosol treatment with HSBPC–liposomes in aerosol (15 mg/ml in the aerosol generator reservoir) and later to a 10-fold higher dose. Pulmonary function studies revealed no adverse effects and the liposome aerosol treatment was otherwise well tolerated. Knight, C.M. *et al.* (1994) exposed 10 volunteers to inhalations of DLPC–liposomes (25 mg/ml) followed by Bec–DLPC (1 mg Bec/ml plus 25 mg/ml of DLPC) for 15 minutes each on consecutive days. There were no significant changes in pulmonary function, and laboratory studies and the inhalations were well tolerated.

Liposome-encapsulated drugs have been associated with reduced toxicity *in vitro* and *in vivo* in a number of other experimental systems. Joly *et al.* (1992) cited reduced renal toxicity in rabbits and reduced renal tubular cell toxicity *in vitro* of amphotericin B liposomes. Wyde *et al.* (1988) found a greater than 10-fold reduction in toxicity to multiple cell culture lines of enviroxime in a liposomal formulation compared with the drug in the absence of liposomes. Furthermore, drug–liposomes may prevent local irritation and reduce toxicity both locally and systemically (Juliano & McCullough, 1980; Smeesters *et al.*, 1988; Aguado *et al.*, 1993; Schrier *et al.*, 1993). Increased potency with reduced toxicity is characteristic of many drug–liposomal formulations (Cullis *et al.*, 1989). It is likely that these findings will apply to aerosolized drug–liposomes; however, this remains to be determined in clinical studies.

GC–liposome aerosols

Studies from our laboratory have demonstrated that five different topically potent, hydrophobic GC, currently employed for asthma therapy with MDI/DPI, can be formulated into stable liposomes suitable for aerosol therapy (Waldrep *et al.*, 1994c). From these studies we have chosen DLPC as the most suitable for GC–liposome aerosols, although other natural or synthetic PC could be substituted, including HSBPC. It is important to employ ultrapure phospholipids to form well-defined and stable liposomes. Encapsulation of GC into liposomes increases the aerosol GC output from most jet nebulizers two to five times over that of microcrystalline GC suspensions of Becotide and Pulmicort, which nebulize with very low efficiency (Waldrep *et al.*, 1994a). GC–liposome aerosol formulations also possess another advantage over current microcrystalline suspensions, namely, particle sizes from selected nebulizers and in the 1–2 μm size range (Waldrep *et al.*, 1993), whereas Becotide particles are reported to be 3.7 μm in diameter and Pulmicort is 2.4 μm (Edman, 1994). The low aerosol output (which is nebulizer dependent) and large particle size may explain the inconsistent clinical efficacy, particularly with Becotide (Bisgaard, 1994).

By using the computer model of particle size and measured output as described, patterns of respiratory tract deposition of GC–liposome aerosols can be estimated. Table 41.7 shows the size characteristics of aerosols of five GC–DLPC liposomes. The average aerosol GC output was 21–22 μg/l of aerosol, an amount suitable for delivering a daily GC dose in 15 minutes or less (based on MDI/DPI daily dosages). The potential increased efficacy of GC–liposomes over microcrystalline GC particles deposited within the airways could lead to reduced dosages (or shorter treatment periods).

CsA–liposome aerosol

Alexander *et al.* (1992) found that oral CsA (5 mg/kg) produced clinical benefit in asthmatics. Prolonged systemic use of CsA, however, seems unacceptable, due to its systemic toxicity. The promising results of this work suggest that aerosolized CsA may prove to be an effective alternative to conventional delivery systems for the treatment of asthma. The use of CsA in aerosol with ethanol as a solvent was examined by Detwiler *et al.* (1990) in rats and

Table 41.7 Size characteristics of five hydrophobic glucocorticoid DLPC liposome aerosols*. (Modified from Waldrep, 1994c.)

Drug	MMAD† (μm)	GSD†
Budesonide	1.7	2.4
Beclomethasone dipropionate	1.6	2.1
Flunisolide	1.7	2.5
Triamcinolone acetonide	1.6	2.6
Dexamethasone	1.6	2.4

* Puritan Bennett twin jet nebulizer modified to single jet.
† Particle size and GSD were based on samples obtained in the Andersen Cascade Impactor.
See text for definition of abbreviations.

in dogs by Dowling *et al.* (1990). In the Detwiler *et al.* (1990) study, no abnormalities of lung histology were detected and CO_2 response curves were not abnormal during a 15-day study. In the Dowling *et al.* (1990) study, rejection of canine lung allografts was prevented or reduced by dosages of CsA in which 95% did not reach serum trough levels of 150 ng/ml. No histological evidence of tissue toxicity from CsA was found. In a preliminary human study in patients with severe, advanced allograft rejection, response to aerosol treatment was variable (Duncan *et al.*, 1994), possibly because of differences in delivery of drug to the allograft. Marked differences in regional deposition appeared to explain the variability, as assessed by radioaerosol technique (O'Riordan *et al.*, 1994). Airway irritation (by ethanol) caused the discontinuation of the use of this treatment.

CsA has been prepared in a liposome aerosol formulation (Gilbert *et al.*, 1993; Waldrep *et al.*, 1993). In Table 41.8, data are presented as particle size and drug output for several formulations nebulized with a modified Puritan Bennett 1600 nebulizer. Aerosol CsA output was reduced with dimyristoyl PC (DMPC), egg yolk PC (EYPC) and dipalmitoyl PC (DPPC) liposomes and is probably related to the phase transition temperature above the nebulizer operating temperature of 16°C. Thus, for best results with drug–liposome aerosols, phospholipids with lower phase transition temperature should be selected (Waldrep *et al.*, 1993). A liposome formulation containing CsA, 5 mg/ml and DLPC, 37.5 mg/ml (phase transition temperature −2.0°C) with the Aerotech II nebulizer produced an average aerosol output of 90 µg/l over a 20-minute interval with MMAD of 1.6 µm and GSD 1.9. Estimated deposition with this formulation was 13.5 mg CsA/hour of inhalation (11.25 (minimum volume in litres) × 90 µg × 22.3% (deposition in lung) × 60 minutes = 13.5 mg/hour) (Knight, V., 1994). This dosage level should be sufficient to produce beneficial clinical effects in the asthmatic lung while minimizing local and systemic toxicity.

Aerosol treatment during inspiratory flow

While the foregoing estimates of lung deposition were based on continuous-flowing aerosols, more efficient aerosol delivery can be achieved through the use of breath actuation of air flow to the nebulizer (Wolf & Niven, 1994). Co-ordination between nebulizer flow and inspiration prevents unnecessary generation of aerosol and consequent loss into the environment. Figure 41.5 illustrates the particle size and output of CsA–DLPC liposome aerosol preparation during repeated actuations using an Aerotech II nebulizer. The MMAD produced by 12 5-second actuations was 1.5 µm with a calculated GSD of 1.7. The CsA–DLPC liposome aerosol particles were thus slightly smaller than those produced by continuous flow, and this was apparently related to the smaller numbers of larger particles (>5 µm) produced. Similarly, Fig. 41.6 shows the

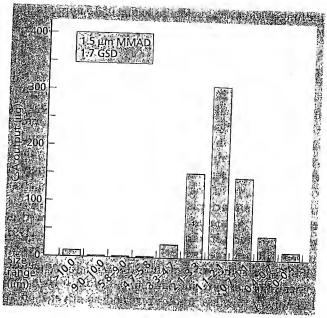

Fig. 41.5 CsA–DLPC liposome aerosol output from Aerotech II nebulizer with manual actuator valve (Pari Jet Interrupter Value System, Munich, Germany); 12 5-second pulses were sampled. Measured output was 59 µg CsA in 22 µl of liquid per actuation. Initial reservoir concentration was 5 mg CsA and 37.5 mg DLPC in combination in liposomes. (Data from J.C. Waldrep, unpublished.) See text for definition of abbreviations.

Table 41.8 Comparison of particle size and output of CsA with six different phospholipids. (From Waldrep *et al.*, 1993.)

	DOPC	DLPC	POPC	DMPC	EYPC	DPPC
MMAD (µm)	1.35	0.82	1.38	1.23	1.29	1.25
GSD	2.31	1.7	2.28	2.27	2.2	3.6*
CsA (5 minutes operation)	654	581	600	979	371	126

* Puritan Bennett twin jet nebulizer with one liquid supply tube removed. CsA to phospholipid ratios 1 : 7.5 (by weight).
See text for definition of abbreviations.

discrete aerosol produced by actuation during inspiration with Bec–DLPC liposomes using the Aerotech II nebulizer, with a resulting MMAD of 1.2 μm and GSD of 1.7. It is thus possible to produce drug–liposome aerosols with similar properties either by actuation during inspiration or by continuous flow to provide efficient alternative methods for the treatment of asthma.

Summary

This chapter, has described aerosol-based therapy, in which aerodynamic particle size is the main determinant for predicting inhaled particle deposition and dosage. By selection of aerosol formulations and delivery systems in which the MMAD is within the range of 1–3 μm and GSD of approximately 2.0, with mouth inhalation, a rather precise targeting of aerosol to the peripheral conducting airways and the alveolated area of the lung is possible. This methodology applies to aqueous aerosols through the usual ranges of salinity and to non-hygroscopic aerosols. A review of studies indicating the feasibility of treatment of asthma with drug–liposome aerosols containing GC and CsA is presented. The authors believe that this methodology has applicability for administration of a wide range of medications for asthma and other pulmonary diseases.

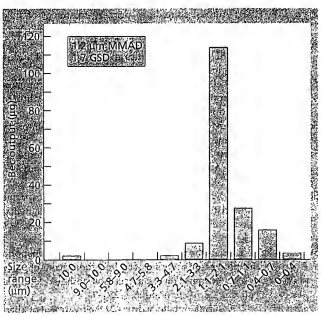

Fig. 41.6 Bec–DLPC liposome aerosol produced by 50 5-second pulses from the nebulizer. Shown is the amount of Bec recovered on the 10 stages of the Andersen Cascade Aerosol Sampler according to particle size. The actuations were controlled by the Pari Jet Interrupter valve system (Munich, Germany). Calculated output per actuation was 3 μg in 72 μl. The reservoir of the nebulizer contained 500 μg/ml of beclomethasone in liposomal formulation. See text for definition of abbreviations. (Data from J.C. Waldrep, unpublished.)

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EXHIBIT 2

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ORIGINAL ARTICLE

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Anticancer exffect of 9-nitrocamptothecin liposome aerosol on human cancer xenografts in nude mice

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Abstract *Purpose*: To test the anticancer properties of the water-insoluble derivative of camptothecin, 9-nitrocamptothecin (9-NC) against human breast, colon and lung cancer xenografts in nude mice when administered in liposome aerosol. *Methods*: The drug was formulated with dilauroylphosphatidylcholine and nebulized in a particle size of 1.6 μm ± 2.0 mass median diameter to deliver doses of usually less than 200 μg/kg daily, 5 days per week. 9-NC liposome aerosols were generated with a Aerotech II nebulizer (CIS-USA) flowing at 10 l/min from a compressed air source and delivered to mice in sealed plastic cages or in a nose-only exposure chamber. *Results*: Tumor growth was greatly reduced or tumors were undetectable after several weeks of treatment. Colon tumor was least responsive. 9-NC was better than the parent compound, camptothecin, also water-insoluble, tested by aerosol in a similar liposomal preparation. Equivalent doses of 9-NC liposome preparations administered by mouth were substantially without effect while there was some effect, but limited, of the liposome preparation given intramuscularly. *Conclusions*: 9-NC liposome aerosol was strikingly effective in the treatment of three human cancer xenografts growing subcutaneously over the thorax in nude mice at doses much smaller than those traditionally used in mice administered by other routes.

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Introduction

Camptothecin (CPT) is a plant alkaloid first isolated from *Camptotheca acuminata* in 1966. As a topoisomerase I inhibitor, it has powerful anticancer properties [5, 16, 33] and has been used clinically in the treatment of a variety of cancers. It possesses significant toxicity, especially involving the bone marrow and gastrointestinal tract that has limited its use. Pantazis has recently reviewed this subject [27].

Derivatives of 20-(*S*)-CPT have been made to increase the aqueous solubility of these compounds and/or to modify the A-ring to increase membrane association. The 9-nitrocamptothecin (9-NC) derivative used in the present study is insoluble in water, but has demonstrated potent antitumor effects against human ovarian and malignant melanoma cells in the human xenograft-nude mouse model when administered intramuscularly at doses in the range 1–4 mg/kg per day [17, 24]. Following studies of oral administration of 9-NC as a dietary supplement [16], the same workers have also found that direct injection of cotton seed oil suspensions of 9-NC through the abdominal wall into the stomach at a dosage of 1.0 mg/kg per day 5 days per week for several weeks, is effective against several human tumor xenografts [25, 26]. A lower dose, 0.75 mg/kg per day given as above, is effective against some more sensitive tumors. In humans, oral treatment with 9-NC at a dosage of 1.0 mg/m^2 per day can be given safely for extended periods, but dose-limiting toxicities occur, especially myelosuppression [22].

Previous work in this laboratory has shown that certain drugs delivered to the respiratory tract in a liposome formulation may have advantages of noninvasive administration, including high pulmonary concentrations, often rapid entry into the systemic circulation, reduced toxicity and reduced dosage requirement compared to oral and parenteral adminis-

tration [11, 12, 29]. Burke et al. [3, 4] prepared liposomal formulations of CPT, 9-NC and other water-insoluble derivatives and Daoud et al. [6] described a potent antitumor effect of these preparations in malignant xenografts in nude mice. With other drug-liposome formulations, we have demonstrated the effectiveness of aerosol administration for the treatment of pulmonary disease as well as systemic disease [13–15]. We have also evaluated the safety and tolerability of the phospholipid used in the preparation of the 9-NC liposomes, dilauroylphosphatidylcholine (DLPC). Rats exposed to 1 h of continuous aerosol for 28 consecutive days show no effect of the phospholipid [10]. Phase I/II studies in humans with DLPC aerosol have also demonstrated its safety and tolerability [1, 31].

In this report we describe the beneficial effect of a liposome aerosol containing 9-NC on xenografts of human breast, colon and lung cancer in nude mice at doses that are considerably smaller than those in the studies described above.

Materials and methods

CPT, 9-NC and other chemicals

20-(*S*)-(CPT) and its derivative 9-NC were gifts from Dr. Beppino C. Giovanella of the Stehlin Foundation for Research, Houston, Tx. CPT was highly purified according to FDA regulations [22]. 9-NC was synthesized from CPT by Dr. Giovanella and was > 99% pure as determined by HPLC analysis and has been given orally to patients [22]. DLPC was purchased from Avanti Polar Lipids, Alabaster, Ala., tertiary butanol (*t*-butanol) from Fisher Scientific, Houston, Tx, and sterile, pyrogen-free water for injection from Baxter Healthcare Corporation, Deerfield, Ill.

Nude mice

Swiss immunodeficient nude mice of the NIH-1 high-fertility strain, bred and housed at the Stehlin Foundation for Research, were used for the experiments [16, 23]. Tumors were implanted at the Stehlin Foundation for Research and the mice were housed at Baylor College of Medicine for treatments.

Human cancer xenografts

Human breast cancer (CLO; infiltrating duct carcinoma), human colon cancer (SQU; moderately differentiated adenocarcinoma) and human lung cancer (SPA; adenocarcinoma) cells were stored, grown and implanted into nude mice as previously reported [16, 18, 23]. Approximately 50 mg (wet weight) of finely minced tumor in 0.5 ml Eagle's minimum essential medium was injected under the skin over the right dorsal chest region. The animals were started on treatment with the experimental drug about 1–4 weeks after implantation of tumors. The sizes of breast tumors in one study were measured in two dimensions (area) with calipers. The sizes of all other tumors were measured in three dimensions (volume). When identifiable, individual tumor masses were measured separately and total area or volume recorded.

Preparation of liposomes containing CPT and 9-NC

CPT (10 mg/ml) and 9-NC (100 mg/ml) were first dissolved in DMSO and heated to 70 °C and 40–50 °C, respectively, to completely solubilize the drugs prior to addition to the phospholipid. DLPC (100 mg/ml) was dissolved in warmed *t*-butanol. Drug and phospholipid preparations, held at 40–50 °C, were mixed at a ratio of 1:50 (w/w). The volume of DMSO in the total organic mixture did not exceed 3–5%. Mixing was performed at room temperature. The material was distributed into 30-ml Wheaton vials (Fisher Scientific, Houston, Tx.), frozen in liquid nitrogen and lyophilized overnight or until thoroughly dried. After sealing the vials under vacuum, the material was stored frozen at –20 °C. For use, sterile, pyrogen-free water for injection was added to the vials to provide the desired concentration of drug. The suspension was gently vortexed until a homogeneous suspension was produced and then transferred to the reservoir of an Aerotech II nebulizer (CIS-USA, Bedford Mass.). The aqueous liposome aerosol suspension, 5–10 ml as needed, was added to the reservoir of the nebulizer. It is necessary to initiate nebulization of the liposomal formulation immediately after dispersing the liposomes in distilled water in order to ensure maximal output of 9-NC in the aerosol. Material left standing at room temperature shows reduced output of 9-NC apparently related to alterations in drug-liposome interactions.

Percoll gradient analysis of efficiency of incorporation 9-NC and CPT into liposomes

9-NC-DLPC liposomes (100–200 µl of liposome suspension) dispersed in water were carefully layered on top of 2 ml Percoll (1.130 g/ml; Sigma, St. Louis, Mo.) and centrifuged at 2000 rpm for 15 min [23]. Liposomes with incorporated drug collected at the top of the Percoll interface while unincorporated drug was deposited at the bottom of the centrifuge tube. The supernatant fraction was carefully removed from the tube and the pellet was resuspended in 200–500 µl acetonitrile. The concentration of 9-NC in an aliquot of the pellet fraction (10 µl) was determined by HPLC analysis and the incorporation efficiency (IE) was calculated as follows: IE (%) = $[(A_T - A_P)/A_T] \times 100$, where A_T is the amount of drug in the original suspension and A_P is the amount of drug in the pellet after centrifugation.

Aerosol treatment

Aerosol was administered to groups of mice in clear, sealed plastic cages (11 × 7 × 5 in). Aerosol was supplied from the Aerotech II nebulizer flowing at 10 l/min via a 1-cm (i.d.) accordion tubing connected to an opening in one end of the cage; aerosol was discharged from an opening in the opposite end of the cage. In one experiment, a nose-only exposure chamber was used (Small Animal Exposure Chamber System, In-Tox Products, Albuquerque, N.M.). The nose-only exposure chamber was used to prevent ingestion of drug by licking or grooming activities by the mice.

Calculation of aerosol dosage of CPT derivatives to mice

Based on estimates of the minute volume [28], mice will exchange 1 l-min/kg of body weight of air (*ca.* 30 ml/min for the nude mice used in these studies) and it is estimated that mice will deposit about 30% of the inhaled particles [8]. It is estimated that one-half to two-thirds of the inhaled particles will deposit in the nose and head of the mouse. These particles and those deposited in the upper conducting airway will be carried promptly by mucociliary action to the orifice of the esophagus and swallowed. Depending on the drug, some fraction may be absorbed through mucus membranes in the nose, head and upper airway. Some 10–15% of the inhaled particles will deposit in the peripheral lungs. The estimated deposited dose (µg/kg) of 9-NC in mice was calculated by multiplying the concentration of 9-NC in the aerosol (µg/l) by the minute volume (l-min/kg), duration of treatment (min) and the estimated deposited fraction. A range of dosage calculations for 9-NC is shown in Table 1.

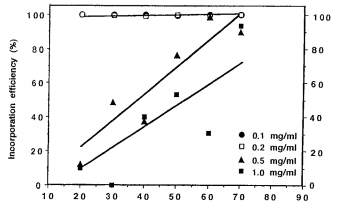

Fig. 1 Efficiency of incorporation of 9-NC into liposomes composed of DLPC as a function of drug: lipid ratio and concentration in water as determined by Percoll gradient analysis

Table 2 Percoll gradient analysis of 9-NC-DLPC liposome aerosol for presence of 9-NC Crystal

9-NC (mg/ml)	Ratio 9-NC:lipid	Crystals % ± SD
0.2	1:55	9.9 ± 5.0
0.2	1:60	16.4 ± 6.0
0.5	1:50	7.9 ± 6.4
0.5	1:60	7.9 ± 3.0

Material recovered from the Andersen sampler after nebulizing the 0.5 mg/ml 9-NC and 25 mg/ml DLPC liposome preparation showed a close correspondence of these constituents on the eight stages of the sampler (R^2 = 0.831). Similar values were found for lower concentration liposome preparations. The possibility of nebulization of 9-NC crystals not in a liposome formulation was explored by collecting AGI samples when 5 mg/ml 9-NC dispersed in 10 ml water was nebulized for 30 min. For comparison, the liposomal formulation with the same amount of 9-NC was nebulized similarly. Less than 1 µg of 9-NC was recovered from the drug-alone preparation, but 1270 µg was recovered from the liposome formulation.

Effect of nebulization and AGI sampling on liposome stability

9-NC-DLPC liposomes, 0.2 mg/ml 9-NC at 1:50 and 1:60 drug/lipid ratios, and 0.5 mg/ml 9-NC at the same drug/lipid ratios were each nebulized for 15 min in the Aerotech II nebulizer, 10 l/min. The total output of aerosol was collected for the various preparations in the AGI and the suspensions were then analyzed for the presence of crystals by Percoll gradient. Each preparation was analyzed in triplicate. The mean and standard deviations of the four preparations are shown in Table 2.

The small percentage of crystals from the liposomes was probably related to the shear forces associated with nebulization and from collection in the AGI. Both procedures are associated with cooling to 16–17 °C, which may have also contributed to the release of crystals. It is unlikely that free crystals were nebulized as noted above.

Electron microscopy

Aerosol containing 9-NC-DLPC liposomes was collected in an AGI containing distilled water and submitted for electron microscopic examination (Dr. Thomas Giddings, University of Colorado, Boulder, Colo.). The results of 1% uranyl acetate staining of the material are shown in Fig. 2. The 100-nm scale bar on Fig. 2 indicates that most of the multilamellar structures revealed were 100 nm or more in diameter. Much smaller particles, probably representing pieces of bilayer, were seen throughout the field.

Measurement of the size of liposomes

Samples were obtained from the reservoir of the Aerotech II nebulizer at the start, and at 17 and 30 min. Figure 3 shows the mean diameter of the liposomes at these time-points. The mean size at the beginning of nebulization was 2539 ± 91 nm, while at 17 and 30 min the diameters were reduced in size to <400 nm as a result of the shear effect of continuous cycling of the liposome suspension through the nebulizer which occurs within minutes.

Treatment of mice with xenografts of human breast cancer

In the initial experiment, six mice were started on treatment 25 days after xenograft implantation, while five implanted mice served as controls (Fig. 4, Table 3). Treatment consisted of 15-min inhalations of 9-NC-DLPC liposome aerosol (containing 1.8 µg/l 9-NC generated producing an estimated deposited dose of 8.1 µg/kg per day) for 5 days per week. ANOVA indicated a statistically significant difference between the two groups (P < 0.0001). There was an immediately discernible difference in the percent increase in tumor size compared to day 0 of treatment in the two groups of mice (initial mean ± SD tumor size, 90.7 ± 97.2 mm^2), with a rapid increase in mean tumor size in control mice (27.2% per day) and a sevenfold slower increase in the mean size in the treated mice (3.8% per day). The difference was statistically significant (P < 0.05; Student's t-test, two-tailed) by day 8 and continued through day 31 when the experiment was stopped because of the large size of the tumors and necrotic lesions in the untreated animals.

At that time the one control mouse and the two treated mice were saved for further study (Fig. 5). No treatment was given from day 32 to day 47. At that time,

Table 1 9-NC-DLPC liposome aerosol characteristics and estimated dosage in mice. Mass median aerodynamic diameter (*MMAD*) and aerosol concentrations were determined using the Andersen cascade impactor. Dosage Calculations were based on a 30-g mouse with a minute volume of 1 l-min/kg of body weight [28], an average aerosol retention factor of 30% [8] and the aerosol concentration as indicated

MMAD (μm)	9-NC in		Treatment (min/day)	Dosage		
	Reservoir (μg/ml)	Aerosol (μg/l)		μg/kg/min	μg/kg/day	μg/day
0.8	100	1.8	15	0.54	8.1	0.24
0.8	100	1.8	30	0.54	16.1	0.48
1.6	200	3.6	30	1.08	32.4	0.97
1.2	500	8.5	15	2.56	38.3	1.15
1.2	500	8.5	30	2.56	76.7	2.30
1.2	500	8.5	60	2.56	153.4	4.60
1.2	500	8.5	120	2.56	306.7	9.20
1.5	1000	15.9	15	4.76	71.4	2.14
1.5	1000	15.9	30	4.76	143.1	4.29

Drug-DLPC aerosol and liposome particle size determinations

The particle sizes of aerosols containing CPT-DLPC or 9-NC-DLPC liposomes were measured with an Andersen/ACFM nonviable ambient particle sizing sampler (Andersen Instruments, Atlanta, Ga.) [32]. The concentrations of CPT or 9-NC in the aerosol generated with the Aerotech II nebulizer flowing at 10 l/min was also measured. Samples were collected over a 5-min period of operation. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated using KaleidaGraph 2.0 software (Synergy Software, Reading, Pa.). Liposome particle size was determined from samples in aqueous suspension with a Submicron Particle Sizer Model 370 (NICOMP Particle Sizing Systems, Santa Barbara, Calif.). Data were collected until the percent error was < 1.5.

Sampling of aerosol with the all-glass impinger (AGI)

Aerosol was drawn by vacuum through a calibrated glass tube with the tip 4 mm above a 10-ml volume of water in an AGI at a flow rate of 12.5 l/min. (Ace Glass, Vineland, N.J.). Collected fluids were used for analytical purposes.

Assay of CPTs by high-performance liquid chromatography

A Waters 710B WISP automatic injector and Waters Nova-Pak C18 column (3.9 × 150 mm; Waters, Milford, Mass.) at room temperature were used to quantitate CPT and 9-NC. The mobile phase was composed of 30% acetonitrile and 70% water containing 0.1% glacial acetic acid, and flowed at 1.2 ml/min. CPT was detected using a Waters 470 scanning fluorescence detector set to an excitation wavelength of 370 nm and an emission wavelength of 440 nm. 9-NC was detected using the Waters 440 absorbance detector with monitoring at 254 nm. The data were analyzed with Waters Millenium software.

Assay of DLPC

DLPC was measured by HPLC using a 717 WISP autosampler and a Nova-Pak silica column (3.9 × 150 mm; Waters). The mobile phase consisted of acetonitrile/methanol/10 m*M* ammonium trifluoroacetic acid, pH 4.8 (64:28:8 v/v/v). Peaks were detected with a SEDEX 55 mass evaporator detector (Sedere, Alfordville, France). Samples were dissolved in methanol or ethanol [30].

Statistics

Analysis of variance (ANOVA) was performed using the True Epistat statistical package from Epistat Services, Richardson, Tx. Input for analysis consisted of tumor size (surface area, one experiment, or volume measured by calipers), time of measurement and experimental group. *P*-values are based on the data using fixed factors and unweighted means. Multiple comparisons of the effect of day from start of treatment and/or treatment regimen on tumor size were made. Comparison of mean tumor sizes was performed using Student's *t*-test, two-tailed, which is a part of the statistical software of Microsoft Excel, v. 5.0.

Results

Aerosol characteristics and treatment

The aerosol particle size of 9-NC-DLPC liposomes was determined for 9-NC for drug concentrations ranging from 100 to 1000 μg/ml in the reservoir. As measured with the Andersen cascade impactor, the MMAD and GSD ranged from 0.8 to 1.6 μm and from 1.8 to 2.6, respectively. Aerosol particles with these MMAD characteristics are well suited for pulmonary deposition throughout the respiratory tract [24, 27, 28]. The concentration of DLPC in the aerosol was determined to be approximately 50 times the 9-NC concentration. Over this range of drug, the concentration of 9-NC in the aerosol was directly proportional to the concentration in the reservoir ($R^2 = 0.995$) and ranged from 1.8 to 15.9 μg/l aerosol (see Table 1).

Efficiency of incorporation of 9-NC and CPT into DLPC liposomes

Figure 1 shows the efficiency of incorporation of 9-NC into DLPC liposomes as determined by Percoll gradient analysis. The incorporation efficiency of 9-NC at a concentration of 0.1 or 0.2 mg/ml into DLPC liposomes in a range of drug to lipid ratios of 1:20 to 1:70 (w/w) was 100%. Incorporation of 0.5 mg/ml 9-NC was 78% at a 9-NC:DLPC ratio of 1:50 and was 100% at 1:60. Incorporation of 1.0 mg/ml 9-NC was lower but was above 90% at a 9-NC:DLPC ratio of 1:70. Microscopy with polarized light show a few crystals of 9-NC in all preparations, but many more where incorporation was low. This was quantitated by Percoll gradient analysis (Table 2). CPT was less efficiently incorporated but at a CPT to lipid ratio of 1:50 (w/w), concentrations of CPT of 0.1 to 0.5 mg/ml showed 80% or more incorporation (data not shown).

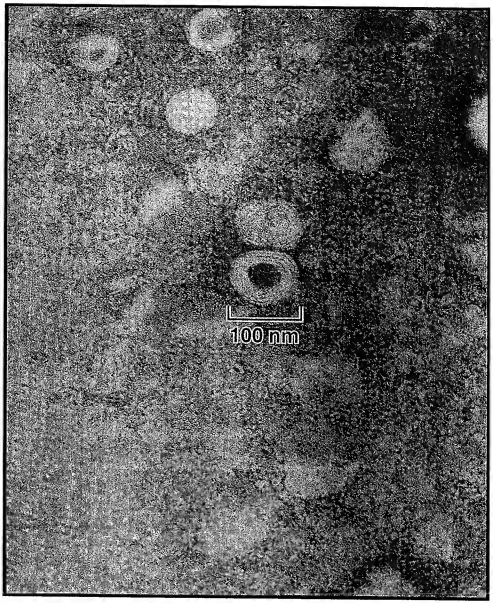

Fig. 2 Electron micrograph of material collected from an aerosol generated by an Aerotech II nebulizer containing 0.5 mg/ml 9-NC and 25 mg/ml DLPC over a 10-min period of operation. Uranyl acetate negative stain (1%) was used (performed by Thomas Giddings, Ph.D., Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colo.)

the two previously treated mice were again started on treatment, this time receiving 38.3 μg/kg 9-NC per day in a 15-min treatment period for 5 days per week. This treatment was continued to day 83 when treatment was stopped. During this later treatment, tumors disappeared in the two mice. These mice were followed for 75 days further. One mouse remained free of detectable tumor but a small tumor reappeared in the other.

Treatment of mice with xenografts of human colon cancer

Eight mice were started on treatment 12 days after implantation of tumor grafts with 9-NC-DLPC liposome aerosol (containing 8.5 μg/l 9-NC generated producing an estimated deposited dose of 76.7 μg/kg 9-NC per day) administered over a single 30-min period for 5 days per week (Fig. 6, Table 3). A second treatment group of seven mice received a similar dosage for 5 days per week for the first 23 days when the total daily dosage was increased to 153.4 μg/kg 9-NC administered as two daily 30-min treatments (a.m. and p.m.) for 5 days per week during the next 19-day period. At this time the dosage was further increased to 306.7 μg/kg 9-NC per day administered as two daily 60-min treatments (a.m. and p.m.) for 5 days per week for an additional 22-day period. Two other groups of ten mice each with xenografts received either DLPC-only liposome aerosol at the same dosage as that contained in the 9-NC aerosols initially used, or no treatment.

As shown in Fig. 6, there was a pronounced reduction in the rate of increase of tumor size (initial mean ± SD tumor volume, 95.3 ± 42.0 mm^3) in the two treated groups that was highly statistically significant ($P < 0.0001$, ANOVA). The mean maximum rate

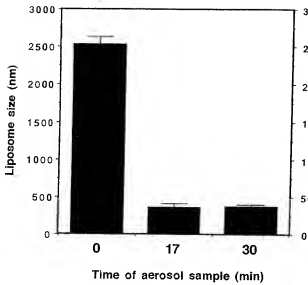

Fig. 3 Particle size of 9-NC-DLPC (1:50, w/w) liposomes. Particle size was determined from samples taken at 0, 17 and 30 min of nebulization from the reservoir of an Aerotech II nebulizer flowing at 10 l/min and containing 0.2 mg/ml 9-NC

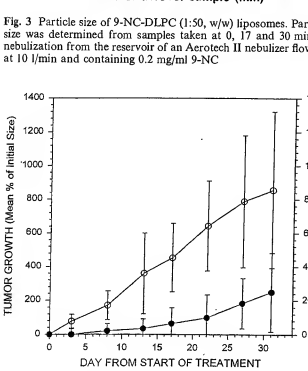

Fig. 4 Treatment of human breast cancer (CLO) xenografts in nude mice with 9-NC-DLPC liposome aerosol. Aerosol was administered for 15 min daily for 5 consecutive days per week for a period of 31 days. The calculated dose was 8.1 μg/kg of 9-NC per day. Values are means ± SD. The mean (±SD) size of tumors at the start of treatment was 90.7 ± 97.2 mm^2 (○ untreated, $n = 5$; ● 9-NC-DLPC liposomes, $n = 6$)

of tumor growth in the two treated groups over the first 42 days of treatment (70.4–113.6 mm^3/day) was seven- to tenfold slower than either the untreated (575 mm^3/day) or the DLPC-only liposome-treated (768 mm^3/day) control groups. The rate of tumor growth in mice receiving the constant low dose of 9-NC (38.3 μg/kg per day) remained at about the same level until day 56 when the size of their tumors increased slightly. However, in the treated group that received the increasing doses, the rate of tumor growth after day 42 decreased from 93.4 mm^3/day to 22.3 mm^3/day for the remainder of the experiment. This decrease in growth rate was statistically significant ($P = 0.012$; Student's t-test, two-tailed).

Treatment of mice with xenografts of human lung cancer

Starting 13 days after implantation, groups of 11 mice with xenografts were treated by aerosol with each liposomal drug regimen (aerosols containing 8.5 μg/l 9-NC generated producing an estimated deposited dose of 76.7 μg/kg 9-NC per day) for 5 days per week, while 11 other mice received no treatment (Fig. 7, Table 3). Initial mean ± SD tumor size at the start of treatment was 457 ± 76 mm^3 and the subsequent mean maximum rate of tumor growth in the untreated mice was 351 mm^3/day. Mean tumor growth was significantly reduced in both treatment groups ($P < 0.0001$, ANOVA), but the effect was greatest in animals receiving 9-NC. The difference in mean tumor volume between the untreated and 9-NC-treated mice was statistically significant ($P < 0.05$; Student's t-test, two-tailed) on day 7 while it required 14 days for the CPT-treated group to achieve significance. At all time-points, the mean size of tumors in the 9-NC-treated group was statistically significantly less than in the CPT group. Five animals in the 9-NC treatment group and six in the CPT treatment group died during the experiment. Most of the animals in the treatment groups developed a skin lesion over their dorsal skin which on histologic examination was found to be a pyoderma with micro-abscesses and many gram-positive cocci resembling staphylococci. Most deaths, either by sacrifice or spontaneous, occurred during the period from day 14 through day 24 of treatment. Later, as the tumor size reduced, the skin lesions disappeared and the animals gained weight and became more active. The increase in mean tumor volume of the CPT-DLPC-treated mice on day 35 compared to that on day 21 was not statistically significant ($P = 0.129$; Student's t-test, two-tailed).

Comparison of oral and aerosol treatment with 9-NC liposomes

A second experimental treatment of nude mice with the same human lung carcinoma was performed with 9-NC (Fig. 8, Table 3). It consisted of 11 mice treated with 9-NC-DLPC liposome aerosol and 11 mice given orally the same liposome preparation. The dose by aerosol was 76.6 μg/kg 9-NC per day for 5 days per week and the oral dose was 100 μg/kg 9-NC per day on the same schedule. Treatment was started on day 15 after xenograft implantation. By this time the tumor sizes were larger than in previous experiments (initial mean ± SD tumor volume, 760 ± 527 mm^3). Because of little apparent effect on the initial mean maximum rate of tumor growth (246 mm^3/day), the doses of both aerosol and

Table 3 Summary of the survival of nude mice during treatment with 9-NC and CPT

Experimental group	Liposome treatment	No. of mice		Duration of study (days)	No. of mice died or sacrificed (day)	Explanation[b]
		Start of experiment	End of experiment			
Breast cancer (CLO)	9-NC aerosol	6	5	31	1 (24)	Sacrificed for histology
	No treatment	5	3		2 (17)	Histology (1); died (1)
Continuation of above experiment:[a]						
Breast cancer (CLO)	9-NC aerosol	2	2	83	0	Large tumors
	No treatment	1	1		1 (51)	
Colon cancer (SQU)	9-NC aerosol, constant dose	8	6	63	2 (14, 37)	Large tumors
	9-NC aerosol, variable dose	7	7		0	
	DLPC only aerosol	10	0		3 (23), 4 (28), 3 (33)	Large tumors
	No treatment	10	0		1 (14), 2 (23), 4 (28), 3 (33)	Large tumors
Lung cancer (SPA)	9-NC aerosol	11	5	36	1 (11), 2 (14), 2 (17), 1 (24)	Emaciation, sacrificed or spontaneous death[c]
	CPT aerosol	11	6		2 (14), 2 (17), 1 (35)	As above
	No treatment	11	0		1 (24), 8 (28), 2 (32)	Large tumors
Lung cancer (SPA)	9-NC aerosol	11	9	49	1 (30), 1 (33)	Eye injury, emaciation
	9-NC oral	11	0		11 (33)	Large tumors
	No treatment	11	0		1 (19), 10 (33)	Large tumors
Lung cancer (SPA)	9-NC aerosol (nose-only)	8	7	36	1 (1)	Accident
	9-NC intramuscularly	8	8		0	
	No treatment	8	8		0	

[a] One of the three surviving untreated, and two of the five surviving 9-NC aerosol-treated mice were followed further at an increased dosage. The other mice were sacrificed for toxicity studies
[b] Animals were sacrificed due to large and/or necrotic tumors
[c] Several of these animals had a gram-positive coccal pyoderma which cleared as tumor size diminished

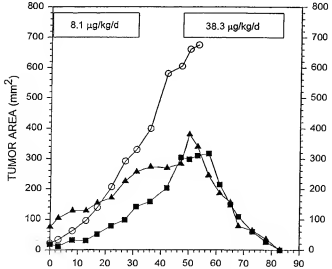

Fig. 5 Treatment of human breast cancer (CLO) xenografts in two nude mice from the experiment described in Fig. 4, followed by no treatment from day 32 to day 47, then treatment with 38.3 µg/kg 9-NC-DLPC per day for 5 consecutive days per week from day 47 to day 83 (○ untreated mouse; ■ mouse #3, ▲ mouse #5)

oral drug were doubled on day 13. Aerosol was administered twice daily for 30 min (a.m. and p.m.). By day 19 the tumors of the aerosol-treated animals were smaller in size than those in the untreated mice ($P = 0.004$; Student's t-test, two-tailed) and the mean rate of growth was decreasing (-138 mm^3/day). This decrease in tumor volume was statistically significant for days 19 through 33 ($P \geq 0.004$; Student's t-test, two-tailed). The tumors animals receiving oral dosage continued to increase in size in a closely comparable manner to those of the untreated group. By day 40, tumors in the aerosol-treated animals had regressed in size nearly to that at the start of treatment. The difference in mean tumor size between aerosol-treated animals and the other two groups of animals was highly significant ($P < 0.0001$, ANOVA). Of the nine 9-NC-DLPC-treated mice surviving on day 49, four did not have any measurable tumor mass. The increase in mean tumor volume in the 9-NC-DLPC aerosol-treated mice which occurred after treatment was stopped was not statistically significant ($P = 0.480$; Student's t-test, two-tailed). This increase was due to the fact that two of the mice had large tumors which had not decreased in size as rapidly as those in the other animals, suggesting that longer treatment might have been more effective.

Nose-Only 9-NC liposome aerosol treatment of mice with xenografts of human lung cancer

A third experimental treatment of nude mice with human lung carcinoma was conducted to evaluate the

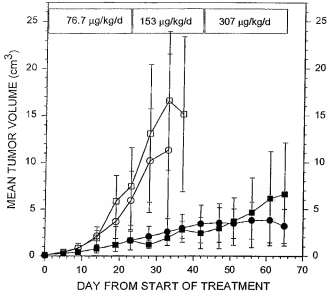

Fig. 6 Treatment of human colon cancer (SQU) xenografts in nude mice with 9-NC-DLPC liposome aerosol. The following dosing schedules were used: 76.7 µg/kg 9-NC per day for 5 consecutive days per week from day 0 to day 62 (constant dose): or 76.7 µg/kg 9-NC per day for 5 consecutive days per week from day 0 to day 23, 153.4 µg/kg per day twice daily for 5 consecutive days per week from day 24 to 42, and 306.7 µg/kg per day twice daily for 5 consecutive days per week from day 43 to day 65 (increasing dose). Values are means ± SD (○ untreated, $n = 10$; □ DLPC only liposomes, $n = 10$; ■ 9-NC-DLPC liposomes, constant dose, $n = 8$; ● 9-NC-DLPC liposomes, increasing dose, $n = 7$)

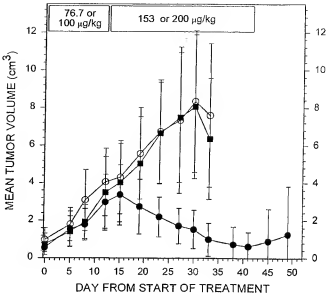

Fig. 8 Treatment of human lung cancer (SPA) xenografts in nude mice with 9-NC-DLPC liposome aerosol or oral administration. The aerosol dosage was 76.7 µg/kg 9-NC per day for 5 consecutive days per week from day 0 to day 12. The dose was increased to 153.4 µg/kg per day twice daily for 5 days per week from day 13 to day 41. The oral 9-NC-DLPC liposomes in aqueous suspension were administered at 100 and 200 µg/kg 9-NC per day following the same regimen as aerosol treatments. Values are means ± SD (○ untreated, $n = 11$; ■ oral 9-NC-DLPC liposomes, $n = 11$; ● aerosol 9-NC-DLPC liposomes, $n = 11$)

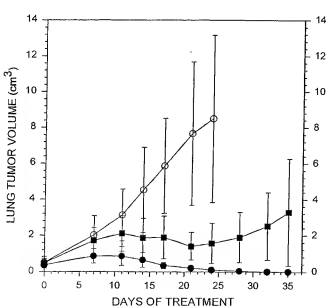

Fig. 7 Treatment of human lung cancer (SPA) xenografts in nude mice with 9-NC-DLPC or CPT-DLPC liposome aerosol. The liposome aerosols were administered in a dosage of 76.7 µg/kg 9-NC or CPT per day for 5 consecutive days per week from day 0 to day 35. Values are means ± SD (○ untreated, $n = 11$; ■ CPT-DLPC liposomes, $n = 11$; ● 9-NC-DLPC liposomes, $n = 11$)

effect of nose-only aerosol exposure or intramuscular injection of 9-NC-DLPC liposomes on tumor growth. Nose-only aerosol exposure was used to evaluate the possible effect of drug consumed orally as a consequence of animal grooming. Each group consisted of eight mice and were untreated, given a single intramuscular injection into the hind leg of 100 µg/kg 9-NC per day on days 0–23 followed by 200 µg/kg per day on days 24–36, or given 77 µg/kg 9-NC per day by nose-only aerosol exposure on days 0–23 followed by 153 µg/kg per day twice daily on days 24–36. Treatment was started 8 days after xenograft implantation and administered for 5 days per week. The mean initial tumor size was 96.8 ± 65.0 mm^3.

By day 19, mean tumor volumes were significantly different in the three groups (Fig. 9, Table 3; $P < 0.001$, ANOVA). While intramuscular injection was moderately effective compared to no treatment from day 19 on ($P < 0.02$; Student's t-test, two-tailed), this treatment was not as effective as the nose-only aerosol exposure ($P < 0.04$; Student's t-test, two-tailed). Following 12 days of aerosol treatment, tumor volume was statistically significantly less than in the untreated controls ($P \leq 0.014$; Student's t-test, two-tailed). Mean tumor growth rate in the aerosol treatment group was more than seven times slower than in the untreated controls from day 8 through 23 (57.9 mm^3/day vs 441 mm^3/day, respectively).

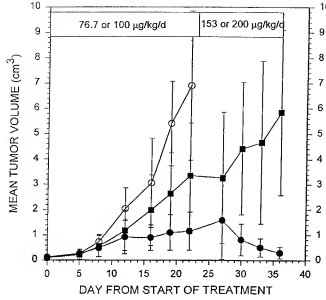

Fig. 9 Treatment of nude mice with human lung cancer (SPA) xenografts with 9-NC-DLPC liposomes administered by aerosol using a nose-only exposure device or by intramuscular injection. Values are means ± SD (○ untreated, $n = 8$; ● aerosol, 76.7 μg/kg 9-NC per day, 5 days per week from day 0 to day 23, then dosage doubled from day 24 to day 36, $n = 8$; ■ intramuscular injections into the hind legs, 100 μg/kg 9-NC per day, then 200 μg/kg per day on same schedule as aerosol treatment, $n = 8$)

Discussion

These studies showed a potent anticancer effect of 9-NC incorporated into DLPC liposomes and administered as an aerosol to nude mice implanted with three different human cancer xenografts (breast, colon and lung). The daily estimated doses associated with easily detectable suppression of tumor growth ranged from 8.1 μg/kg 9-NC per day in a 15-min inhalation to as high as 306.7 μg/kg per day in multiple inhalation periods. Most doses were 38.3 to 76.7 μg/kg per day in a single treatment period. Treatments ranged from 15 to 60 min in duration and were administered five times per week over periods of several weeks to several months. Since oral and intramuscular administration of the liposome formulations at comparable doses was only marginally effective at best, the substantial benefit of the liposome preparation administered by aerosol suggests a major role of this route in producing the favorable response.

We found that incorporation of 9-NC and CPT into DLPC liposomes was nearly complete when first formed. However, after nebulization, a small percentage of crystals was found in aerosols sampled using the AGI. There was no evidence of pulmonary toxicity in this study and thus there were apparently no untoward effects of the small proportion of crystals that may have been inhaled.

In our studies, treatment was continued long enough to establish a statistically significant difference in tumor size between treated and control groups. Since tumor size was receding in most treatment groups at the close of the experiments, further treatment would likely have resulted in greater regression in size of tumors. The least favorable results were with animals xenografted with human colon cancer. With this aggressive tumor (highest growth rate of those tested), longer treatments may be needed. This was suggested by the stabilization of growth in the group treated with an increased dose after day 42 (Fig. 6).

For comparison, investigators at the Stehlin Foundation [16, 24, 25] have used 9-NC by intramuscular injection of 1.5–2.0 mg/kg twice weekly for 3–4 weeks, producing complete remissions in colon, lung and breast cancer, malignant melanoma and ovarian carcinoma xenografts in nude mice. In some experiments partial remissions occurred. There were very few treatment failures. The authors report that the above dosage is about the largest reasonably well-tolerated dose in this model, and that lower doses are not effective. In comparison, the largest dose we used in these studies was only about 1/5 or less of the intramuscular dose on a weekly basis. The doses we used were also smaller than the generally effective dose of 1.0 mg/kg per day given 5 days per week for several weeks by direct injection into the stomach of mice with human cancer xenografts cited earlier in this report.

We examined lungs and other organs of a number of mice involved in this study, many after a month or more of treatment. None of the histological sections showed evidence of drug toxicity. Likewise, bone marrow from 16 treated mice was examined and no findings indicated myelosuppression (unpublished results).

As described previously, about 30% of aerosol inhaled by mice is deposits in the respiratory tract; the remaining 70% is exhaled [8]. At least one-half of the deposited aerosol is found on mucus membranes of the nose, head, trachea and upper bronchi. Particles deposited at these sites are promptly transported to the esophageal orifice and swallowed. Despite this distribution of inhaled particles, as shown above, only aerosol treatment was effective, implying that it was the pulmonary deposition that was essential. We found (unpublished observations) that drug deposited in the lungs by inhalation was transported in a few minutes to the systemic circulation and accumulated principally in the liver and to a lesser extent in the spleen and kidney. Drug was found also in the tumor tissue.

The use of 9-NC-DLPC liposome aerosol in humans has advantages over its use in mice. About 70% of inhaled aerosol will be deposited by nasal breathing in an adult human [20, 21]. More than one-half of this amount will be deposited in the nasopharynx. A large fraction of this deposition will be transported to the esophageal orifice and swallowed. In addition, some of the drug deposited in the trachea and upper bronchi will be transported upwards and swallowed. Alternatively, the aerosol can be administered by mouth breathing. Less than 5% given by this route will be deposited in the mouth and about 27% of the total inhaled amount will

be deposited in the lungs. As in rodents, some material deposited in the trachea and upper bronchi will be transported upward and swallowed, but the majority of the dose deposited in the lungs will remain there to diffuse into the systemic circulation. Thus, administration of aerosol particles to the lungs is more efficient in humans than in mice.

Other differences between the nude mouse-xenograft models of human cancer and the treatment of cancer in humans may be significant. Besides the benefit of pulmonary administration cited above, the greater inactivation of the CPTs by human serum albumin [2, 9], the differences in metabolic conversion of 9-NC to 9-amino-CPT [19], the greater myelosuppressive effects in humans [7, 22] and the use of immunodeficient mice raise important questions that can only be answered by studies in humans.

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